Transcriptional Regulation of the Macrophage Inflammatory Protein 1-alpha/ Stem Cell Inhibitor Gene.

by

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This thesis is submitted in part fulfilment of the Degree of Doctor of Philosophy in the University of Glasgow.

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September 1993.

This thesis is dedicated to those who venture to read it. Good luck.

Declaration

Unless otherwise acknowledged, all of the work in this thesis, physical and mental, was performed by me personally.

Acknowledgements.

Firstly, many thanks to Dr. Mark Plumb for his unique style and method of supervision: though we seldom agreed about anything, reaching a compromise was always informative and entertaining, though whoever payed the phone bills may not think so. Secondly, thanks to Dr. Saveria Campo for the discussions and encouragement over the last few years. Thanks also to Ian Pragnell, Gerry Graham and Alan Reid for many interesting conversations about science and cycling, peaked caps and St Mirren. Thanks also to Drs. R.J. Bhutto and Randy Feelups, for what I'm not sure, but thanks anyway. Finally, many thanks to Quentin Longbottom for his continuing willingness to take the flak in adverse situations.

Abstract

Macrophage inflammatory protein 1α (MIP1α) protein is an inhibitor of haemopoietic stem cell proliferation and an inflammatory mediator. MIP1α protein is present in normal bone marrow, and is believed to be produced there by a sub-population of macrophages. The gene encoding the MIP1α protein is an early-response gene as it is present at low or undetectable levels in uninduced haemopoietic cell-types, and is rapidly inducible by inflammatory stimuli in a haemopoietic cell-specific manner. The presence of high constitutive levels of MIP1α gene expression in fresh haemopoietic tumour cells and in the peripheral blood and/or bone marrow of patients with leukaemic and pre-leukaemic disorders shows that the gene can be disregulated, by an unknown mechanism(s), with potentially profound effects on the haemopoietic system. As gene expression is controlled ultimately at the level of transcription, the work presented in this thesis is an examination of the transcriptional regulation of the MIP1α gene in macrophage cell lines compared to non-MIP1α-expressing cell types, in order to address the questions of how the MIP1α gene is regulated *in-vivo* and by what mechanism(s) its expression can be disregulated.

A positive λEMBL3 genomic clone was characterized and sequenced, confirming the presence of the MIP1α gene. MIP1α gene expression was shown to be rapidly and transiently induced by lipopolysaccharide (LPS), fresh serum and interferon α/β, and to be down-regulated by transforming growth factor beta and interferon-γ in macrophages, confirming that MIP1α is an early-response gene in macrophages, and consistent with a role(s) for macrophage-derived MIP1α protein in wound-healing and inflammation.

A single constitutive nuclear DNase1 hypersensitive site, which maps to the proximal 250-350 bp of the MIP1α promoter, was identified in macrophage cells but was absent in cells which do not express basal levels of MIP1α mRNA. Consistent with this, the promoter sequences from +36 bp to -220 bp are sufficent to confer cell-specific and inducible transcription on a reporter gene in transfection assays. This region consists of proximal and distal transcriptional regulatory elements, both of

which contribute to the cell-specific promoter activity; the proximal region (+36 bp to -160 bp) confers constitutive, LPS- and serum-responsive promoter activity, and the distal region (-160 bp to -220 bp) confers constitutive and serum-responsive promoter activity.

In-vitro DNA-binding studies revealed five major nucleoprotein binding sites in the proximal promoter, which bind C/EBP-, c-Rel and c-Ets family members. Cell-specific differences in DNA binding by a c-Rel family protein-containing complex correlates with the cell-specificty of endogenous MIP1α gene expression and the chromosomal conformation of the promoter. Cell-specific differences in DNA binding by C/EBP-, c-Rel- and c-Ets family members correlates with the cell-specificity of reporter gene expression conferred by the MIP1α promoter in transfection assays. Changes in promoter binding by members of the C/EBP- and c-Rel families correlate with the observed LPS-stimulated transcriptional up-regulation of the endogenous MIP1α gene measured by nuclear run-on analysis-, and serum- and LPS-stimulated up-regulation of proximal MIP1α promoter constructs in transfection assays-, in macrophages.

Functional transfection studies of wild-type and point-mutated MIP1α promoter constructs, together with *in-vitro* promoter binding studies and sequence comparisons show that AP-1 can bind to the distal regulatory element, and suggest that it has a negative-regulatory activity, but that its DNA binding in unstimulated- and LPS-stimulated macrophages is occluded by postive-regulatory factors binding to adjacent promoter sites. Functional transfection studies in macrophages, of point-mutated MIP1α promoters, show that the positive-regulatory activity of the distal promoter element is dependent on the integrity of the distal-most C/EBP family binding site of the proximal MIP1α promoter element; this is supported by *in-vitro* DNA binding studies which suggest that non-AP-1 transcription factor binding to the distal regulatory element is co-operative.

A testable model for the observed cell-specific and inducible transcriptional activity of the MIP1α gene is proposed, based on the *in-vitro* and functional data presented in this study. This model suggests mechanisms by which: 1) the kinetics of

induction of the MIP1α gene in macrophages by different agents is different; 2) TGFβ, IFNγ and prostaglandins negatively-regulate MIP1α gene transcriptional activity in macrophages; 3) cultured macrophage cell-lines may be a good model for inflammatory macrophages but not for tissue-macrophages; 4) tissue macrophages may produce a constitutively low level of MIP1α protein; 5) the MIP1α gene is disregulated in haemopoietic fresh tumour cells; 6) the MIP1α gene may be inducible in certain non-haemopoietic cell-types.

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Abbreviations.

5-Aza	5-azacytidine
5-FU	5-fluorouracil
A	Adenine
A-MuLV	Abelson leukaemia virus
A_n	$Absorbance_{walength\ in\ nanometres}$
AP-1	activator protein-1
APS	ammonium persulphate
ARE	AU-rich instability element
ATP	adenosine triphosphate
b	base
β-gal	beta galactosidase
bHLH	basic region-helix-loop-helix
bHLH-ZIP	basic region-helix-loop-helix-leucine zipper
BM	bone marrow
BMDM	bone marrow-derived macrophage
bp	base pair
BPV	bovine papilloma virus
BSA	bovine seum albumin
bZIP	basic region-leucine zipper
C	cytosine
c-	cellular
C/EBP	CCAAT/ enhancer binding protein
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CFC	colony-forming cell
CFU-A	colony-forming unit assay
CFU-GM	colony-forming unit-granulocyte-macrophage
CFU-S	colony-forming unit spleen
CHX	cycloheximide
Ci	curie
CIP	calf intestinal alkaline phosphate
cpm	counts per minute
CRE	cyclic adenosine monophosphate response element
CREB	CRE binding protein
CTP	cytdidine triphosphate
d12	day twelve
d8	day eight
DAG	diacylglyerol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphoshate
ddNTP	dideoxynucleoside triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphospate

DHS	DNase1 hypersensitive site
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	3' deoxyribonucleoside 5' triphosphate
ds	double-stranded
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid, disodium salt
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
FCS	foetal calf serum
g	gram
G	guanine
G-CSF	granulocyte colony-stimulating factor
GAP	GTP-ase activating protein
GAPDH	glyceraldehyde phosphate dehydrogenase
GAS	interferon gamma activated site
Gi	inhibitory G protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GR	glucocorticoid receptor
Gs	stimulatory G protein
GTP	guanosine triphosphate
HD	homeodomain
Hepes	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
hGH	human growth hormone
HLH	helix-loop-helix
hMIP	human macrophage inflammatory protein
hr	hour
HS	hypersensitive site
HSB	high salt buffer
HSV	herpes simplex virus
HTH	helix-turn-helix
IFNα/β	interferon alpha-beta
IFNγ	interferon gamma
IL	interleukin
Inr	initiator
IP_3	inositol triphosphate
ISRE	interferon-stimulated response element
k	kilo
kDa	kilodaltons
l	litre
LCR	locus control region
LMP	low melting point
LPS	lipopolysaccharide
LSB	low salt buffer

LTBMC	long-term bone marrow culture
LZ	leucine zipper
μ	micro
m	milli
M	molar
M-CSF	macrophage colony stimulating factor
mA	milliamps
MAP	mitogen activated protein
MAR	matrix attatchment region
MCP	monocyte chemotactic protein
MEL	mouse erythroleukaemia
mins	minutes
MIP	macrophage inflammatory protein
mm	millimetre
MMTV	mouse mammary tumour virus
MOPS	3-(N-morpholino) propanesulphonic acid
mRNA	messenger RNA
NaAc	sodium acetate
NF-	nuclear factor
nm	nanometre
NTP	nucleoside triphosphate
^{o}C	degrees celcius
OD	optical density
ONPG	o-nitrophenyl-β-D-galactopyranoside
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC	preinitiation complex
PCR	polymerase chain reaction
PEV	position effect variegation
PF-4	platelet factor four
phage	bacteriophage
PI	phosphatidylinositol
PIP_2	phosphatidylinositol 4,5 bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
pm	picomolar
PMA	phorbol myristal acetate
PMSF	phenylmethyl sulphonyl fluoride
PNPP	p-nitrophenyl phosphate
PP-	protein phosphatase
PTPase	protein tyrosine phosphatase
RANTES	regulated on activation normal T expressed and secreted
RHD	rel homology domain
RNA	ribonucleic acid
RNase	ribonuclease

RPA	ribonuclease protection assay
rpm	revolutions per minute
RSRF	related to serum response factor
RTK	receptor tyrosine kinase
S-phase	DNA synthesis-phase
SAR	scaffold attatchment region
SCF	stem cell factor
SCI	stem cell inhibitor
SDS	sodium dodecyl sulphate
SFFV	spleen focus-forming virus
SHR	steroid hormone receptor
SIS	small inducible secreted
Sl	*Steel* locus
Sl^d	*Steel-Dickie* locus
SPAP	secreted placental alkaline phosphatase
SRE	serum response element
SRF	serum response factor
ss	single-stranded
SSC	sodium chloride and sodium citrate
su(Hw)	supressor of hairy wing
SV40	simian virus 40
T	thymidine
TAF	TBP-associated factor
TBP	TATA binding protein
TCR	T cell receptor
TEMED	tetramethylenediamine
TF-	transcription factor
TGFβ	transforming growth factor beta
TK	thymidine kinase
TNFα	tumour necrosis factor alpha
Topo-	topoisomerase-
TPA	12-O-tetradecanoyl 13-phorbol acetate
TRE	TPA response element
Tris	2-amino-2-(hydroxymethyl) propane-1,3-diol
tRNA	transfer RNA
U	units
UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
V	volts
v-	viral
v/v	volume for volume
w/v	weight for volume
wt	wild-type

PART 1: INTRODUCTION.

CHAPTER 1: THE HAEMOPOIETIC SYSTEM.

1.1. OVERVIEW:

Eight major families of mature haemopoietic cells are currently recognised: erythroid, granulocytic (neutrophilic), eosinophilic, megakaryocytic, mast cell, monocytic, T lymophocyte and B lymphocyte (Metcalf, 1984). Some lymphocytes have a life-span that may approach that of the individual, while red blood cells circulate for about four months and neutrophils for only about eight hours (Golde, 1991). The haemopoietic system consists of vast numbers of mature cells, the numbers of which vary very little in steady state conditions, hence vast numbers of mature cells , of the order of $3.7x10^{11}$ cells per day, must be continually produced in a tightly-regulated manner, simply in order to replace those cells lost due to natural wastage (Dexter *et al*, 1987). In times of stress, for example infection or injury, this production rate can increase more than ten-fold (Golde, 1991).

In an adult mammal, all cells of this system originate from a small number of pluripotent haemopoietic stem cells residing mainly in the bone-marrow, and are produced through a process of proliferation, commitment to a particular lineage, and differentiation. The haemopoietic system has conventionally been described as consisting of three overlapping cell populations: stem cells, progenitor cells and maturing end cells. The population is "pyramidal" in shape, in that stem cells constitute about 0.4% , committed precursors (or transiently amplifying progenitors) about 3% , and morphologically recognisable (but still transiently amplifying by proliferation) maturing end cells more than 95% of the total number of haemopoietic cells found in haemopoietic tissues (Metcalf, 1984; Testa *et al*, 1988).

1.2 MATURE END CELLS.

The mature haemopoietic cell-types have diverse functions, from oxygen-carrying in the blood-stream to host-defence. Further discussion of different mature end cells, such as macrophages, will be entered into in the following text only where relevant.

1.3 PROGENITOR CELL COMPARTMENT.

This compartment consists of cells that are committed to a line of cell-development that will give rise to one, or at most two, mature cell-lineages, by a process of amplification and differentiation (Testa *et al*, 1988). Haemopoietic progenitor cells are not morphologically recognisable (Testa *et al*, 1988) but have been extensively investigated using variations of an *in-vitro* clonogenic soft-gel culture (of bone marrow cells) system (Metcalf, 1984; Bradley *et al*, 1966; Ichikawa *et al*, 1966). This assay system led to the identification of the various committed haemopoietic precursor cells. Colonies are named after the initiating progenitor cell: for example, GM-CFC (granulocyte-macrophage colony-forming cell) is an irreversibly committed progenitor which proliferates and differentiates in the *in-vitro* assay to give rise to a colony containing mature granulocytes and/or macrophages, depending on the stimulus used (Metcalf, 1984; Testa *et al*, 1988).

These *in-vitro* assays allowed the identification of several haemopoietic regulators, originally termed the colony stimulating factors (CSFs), which are glycoprotein molecules essential *in-vitro* for the survival, proliferation, differentiation and maturation of lineage-restricted haemopoietic progenitor cells and the stimulation of mature end-cell functional activity (Metcalf, 1985; Nicola, 1989; Nicola *et al*, 1983). For example, G-CSF (granulocyte colony stimulating factor) allows the formation of granulocytes *in-vitro*, from committed G-CFC precursors (Metcalf, 1984).

To date, more than twenty haemopoietic regulators are known (Metcalf, 1992); functions of these regulators are not completely defined, though it is evident that many of them have diverse functions, and several, for example G-CSF, can act on the mature, progenitor and stem-cell compartments, either alone or in synergy with other haemopoietic regulators (Metcalf, 1984; Nicola, 1989; Ikebuchi *et al*, 1988a; Ikebuchi *et al*, 1988b).

1.4 STEM CELL COMPARTMENT.

1.4.1 Definition of a stem cell.

Pluripotent haemopoietic stem cells are loosely-defined as those bone-marrow cells capable of: i) self-renewal; ii) proliferation and differentiation to form all the mature haemopoietic lineages; iii) the ability to prevent the death of an otherwise

lethally-irradiated animal by reconstituting the haemopoietic system of that animal (Spangrude *et al*, 1988).

1.4.2 Evidence for existence of pluripotent haemopoietic stem cells.

Subjection of a mammal to a high enough dose of irradiation (or cytotoxic drugs) leads to ablation of the haemopoietic system of that animal and rapid death. Death is prevented by injection of donor bone-marrow (BM) cells from a healthy animal (Schofield, 1979). Retroviral marker studies show that multiple haemopoietic lineages in these reconstituted animals are derived from the same initiating cell (Williams *et al*, 1984; Lemischka *et al*, 1986), ie. this demonstrates the existence of stem cells.

A quantitative *in-vivo* assay for the detection of primitive murine haemopoietic cells, termed the CFU-S assay, was developed by Till and McCulloch (Till *et al*, 1961): seven to eight days after the injection of donor BM in the above experiments, macroscopic colonies, termed Colony-Forming Unit-Spleen, day eight (CFU-S d8) are detected on the surface of the spleen. These colonies are clonogenic (Williams *et al*, 1984) and contain mainly erythroid cells, but other lineages are also present
(Magli *et al*, 1981; Curry *et al*, 1967). Twelve days after transplantation, further clonogenic colonies, CFU-S d12, are visible (Till *et al*, 1961). These colonies are larger than CFU-S d8 and contain a greater number of lineages (Magli *et al*, 1981; Curry *et al*, 1967).

Serial transplantation studies reveal that a large proportion of CFU-Sd12 give rise to further CFU-S, though very few CFU-Sd8 exhibit this capacity (Simanovitch *et al*, 1963); furthermore, CFU-Sd12 rescue irradiated recipients. Hence, CFU-Sd12 give rise to multiple lineages, are capable of self-renewal, and can reconstitute the haemopoietic system of lethally-irradiated recipients, ie. CFU-Sd12 are members of the stem-cell compartment (Schofield, 1979; Williams *et al*, 1984; Lemischka *et al*, 1986; Till *et al*, 1961; Magli *et al*, 1981; Curry *et al*, 1967; Simanovitch *et al* 1963).

1.4.3 Hierarchy/ age-structure of the haemopoietic stem-cell compartment-

The differences between CFU-Sd12- and CFU-Sd8 colonies suggest that an age-structure, in terms of pluripotentiality and self-renewal potential, exists within the haemopoietic stem cell compartment (Magli *et al*, 1981; Curry *et al*, 1967;

Simanovitch *et al*, 1963). Further, donor CFU-Sd12 have only short-term (less than four weeks) repopulating ability (Ploemacher *et al*, 1989), and the long-term repopulating ability of donor bone marrow does not correlate with the CFU-S content of that marrow (Ploemacher *et al*, 1988). More primitive cells, termed pre-CFU-S have been identified, which can be separated from CFU-S, and which are responsible for the long-term repopulating ability (Ploemacher *et al.* 1988; Ploemacher *et al*, 1989). However, some short-term repopulating cells are required together with the long-term repopulating cells in order for the survival of the irradiated recipient (Ploemacher *et al*, 1989; Jones *et al*, 1990).

Investigation of the proliferative status of components of the stem cell compartment using cytotoxic drugs reveals that 90% of CFU-S are quiescent in normal bone marrow (BM) (Burgess *et al*, 1983). Further analysis shows that in normal BM, CFU-Sd8 are extremely sensitive, CFU-Sd12 less so, and pre-CFU-S refractory to single *in-vivo* exposure to cytotoxic drugs (Hodgson *et al*, 1982; Lerner *et al*, 1990; Rosendaal *et al*, 1977; Hodgson *et al*, 1979). This suggests that in normal BM, CFU-S d8 are either proliferating or easily-triggered into proliferation, hence may be contributing to steady-state haemopoiesis, while more primitive cells are either quiescent or proliferating extremely slowly and thus contributing little to normal haemopoiesis. In fact, even pre-CFU-S appear to be triggered into rapid proliferation three to five days after cytotoxic drug treatment of a mouse, at which time they are sensitive to the effects of further cytotoxic drug treatment (Harrison *et al*, 1991). Hence, all members of the haemopoietic stem cell compartment can be induced to rapidly proliferate, though the more primitive the cell, the longer it takes for this to occur (see also "Positive regulators" section, below).

1.4.4 *In-vitro* stem cell assays.

In-vitro, in the absence of haemopoietic growth factors, haemopoietic progenitor cells do not survive (Metcalf, 1984; Golde, 1991; Dexter *et al*, 1977; Testa *et al*, 1988; Bradley *et al*, 1966; Ichikawa *et al*, 1966; Metcalf, 1985; Nicola, 1989). However, soft-agar clonogenic assays using combinations of growth-factors can be used to promote the survival, proliferation and differentiation of various members of the haemopoietic stem-cell compartment. These assays are named after the component of the stem-cell compartment they detect. Examples of such assays are:

1.4.4.1 Blast colony-forming cells (Blast-CFC).

Incubation of BM in soft agar in the presence of pokeweed mitogen spleen-conditioned medium leads to the production of colonies containing undifferentiated (blast) cells, after sixteen days (Nakahata *et al*, 1982), and each of these colonies contains multiple CFU-S (Nakahata *et al*, 1982). Replating of these colonies leads to the formation of multi-lineage colonies (Nakahata *et al*, 1982). Blast cell colonies are still formed with marrow from mice that have been treated with cytotoxic drugs (Ikebuchi *et al*, 1987). As the incidence of blast-CFC in the marrow is extremely low (about four per 10^5 bone-marrow cells (Nakahata *et al*, 1982)) and blast-CFCs show self-renewal, give rise to multiple mature blood cell lineages and are insensitive to *in-vivo* cytotoxic drug treatment, they show characteristics of- and may be *in-vitro* detected- pre-CFU-S cells (Lerner *et al*, 1990).

Blast colonies can apparently survive without dividing for two weeks in the absence of exogenously-added growth-factors (Leary *et al*, 1989), and several growth factors acting in synergy are required in order to rapidly induce their proliferation (Ikebuchi *et al*, 1987; Leary *et al*, 1989). This trait is shared with primitive haemopoietic stem cells enriched for by other techniques where the more primitive the cell, the greater the number of haemopoietic growth factors required to stimulate its proliferation (Spangrude *et al*, 1991; Heimfeld *et al*, 1991). This correlates with the *in-vivo* experiments using cytotoxic drugs, discussed earlier (Hodgson *et al*, 1982; Lerner *et al*, 1990; Harrison *et al*, 1991), where primitive haemopoietic stem cells are either very slowly proliferating or are quiescent, and are only induced to proliferate rapidly by prolonged *in-vivo* cytotoxic drug treatment (Harrison *et al*, 1991).

1.4.4.2 CFU-A assay.

This assay, developed by Pragnell et al. (Pragnell *et al*, 1988; Eckman *et al*, 1988), employs the incubation of bone-marrow cells in conditioned media believed to contain GM-CSF, M-CSF and SCF (stem cell factor, a haemopoietic growth factor) (Wright *et al*, 1992). CFU-A are normally quiescent, as measured by cytotoxic drug treatment of mice, and appear to be the *in-vitro* equivalent of CFUS d12 cells (Wright *et al*, 1992).

1.5 THE BONE MARROW STROMAL MICRO-ENVIRONMENT: IMPORTANCE IN THE REGULATION OF HAEMOPOIESIS.

1.5.1 The bone marrow stroma:

Haemopoietic cells are associated with the bone marrow stroma, which consists of several adherent cell-types including fibroblasts, reticular adventitial cells and macrophages, plus stromal cell-derived extracellular matrix (ECM) (Chabannon *et al*, 1992). During regeneration of the haemopoietic system of lethally irradiated mice, injected bone marrow stem cells seed selectively to the haemopoietic tissues, ie. bone marrow (mostly) and spleen (Till *et al*, 1961; Aizawa *et al*, 1988). The mechanism for this homing appears to involve membrane lectins on haemopoietic cells (Aizawa *et al*, 1988), which allow binding to specific glycosaminoglycans on stromal cell surfaces or on stromal cell-derived ECM (Aizawa *et al*, 1988; Tavassoli *et al*, 1990). Loss of adhesion, in the process of differentiation, is associated with loss of haemopoietic cell-surface receptors (Patel *et al*, 1989).

Much of what is known about interactions between stromal cells and haemopoietic cells in the bone-marrow is the result of studies using long term bone marrow cultures (LTBMCs) (Dexter *et al*, 1977), wherein bone marrow is grown on culture dishes under conditions that allow stromal cells to adhere to the dish. This adherent layer is able to support haemopoiesis over a period of many months in the absence of exogenously-added haemopoietic growth factors (Dexter *et al*, 1977), and the differentiated haemopoietic cells and non-adherent CFU-GM are derived largely from a progenitor/stem cell population which is tightly adherent to the adherent stromal cells (Mauch *et al*, 1980); in fact, close contact between the stromal cells/stromal cell-derived ECM and the haemopoietic cells is required for the long-term maintenance of haemopoiesis in this system (Bentley, 1981; Verfaillie, 1992).

1.5.2 Positive regulation of haemopoiesis.

1.5.2.1 Difficulty in detection of known haemopoietic growth factors:

A stimulator(s) of CFU-S proliferation, produced by a sub-population of bone marrow stromal macrophages, and that is present in regenerating bone marrow and in LTBMCs at times when CFU-S are actively proliferating, but not in normal bone marrow (BM), has been partially characterised (Lord *et al*, 1977; Wright *et al*, 1982; Toksoz *et al*, 1980; Lord *et al*, 1976).

Although many of the known haemopoietic growth factors, including those which are essential for *in vitro* haemopoietic colony formation (Metcalf, 1984; Golde, 1991; Dexter *et al*, 1987; Testa *et al*, 1988; Bradley *et al*, 1966; Ichikawa *et al*, 1966; Metcalf, 1985; Nicola, 1989; Metcalf, 1992) can be produced by a variety of cell-types, including macrophages, mast cells, B cells, T cells, fibroblasts and bone marrow stromal cells, upon the appropriate stimulation , it has proven difficult to detect many of the factors in unstimulated stromal cells and in LTBMCs (Dexter *et al*, 1990; Zipori, 1988; Zipori, 1990).

The fact that the haemopoietic growth factors can be induced in the above cell types, that they can affect the functions of mature haemopoietic cells (Metcalf, 1984; Golde, 1991; Dexter *et al*, 1987; Testa *et al*, 1988; Bradley *et al*, 1966; Ichikawa *et al*, 1966; Metcalf, 1985; Nicola, 1989), and that they are readily detectable in multiple tissues but not in normal bone marrow (Metcalf, 1984; Golde, 1991; Dexter *et al*, 1987; Testa *et al*, 1988; Bradley *et al*, 1966; Ichikawa *et al*, 1966; Metcalf, 1985; Nicola, 1989; Dexter *et al*, 1990; Zipori, 1988; Zipori, 1990) has suggested that an important role for these factors may be as an "emergency" response to injury or infection (Dexter *et al*, 1990; Zipori, 1990), and has led some investigators to propose that they do not play any role in steady-state haemopoiesis in the bone marrow (Dexter *et al*, 1990; Zipori, 1990) and that other, unknown regulators are important in this role.

1.5.2.2 Haemopoietic growth factors are involved in the regulation of normal haemopoiesis:

Evidence is now accumulating that the known haemopoietic growth factors do play a role in normal haemopoiesis. Irrefutable evidence for this is available for two of these factors that *are* readily detectable in normal bone marrow and LTBMCs, SCF and M-CSF (Kittler *et al*, 1992). Firstly, osteopetrotic (op/op) mice have severe defects in osteoclast and macrophage formation, and these mice have a mutation in the M-CSF gene coding region (Wiktor-Jedrzejczak *et al*, 1992). The defect is corrected by injection of M-CSF but not by transfer of normal haemopoietic cells, suggesting that bone marrow stromal cells are important in this essential production of M-CSF (Wiktor-Jedrzejczak *et al*, 1992). Secondly, the bone marrow microenvironmental (ie. stromal) defect in "Steel" (Sl/Sl^d) mutant mice that leads to

severe anaemia (Russell *et al*, 1979; Wolf *et al*, 1983) has been shown to be due to mutation of the SCF gene (Williams *et al*, 1990; Copeland *et al*, 1990; Flanagan *et al*, 1990a; Flanagan *et al*, 1990b; Zsebo *et al*, 1990a; Zsebo *et al*, 1990b; Anderson *et al*, 1990). In particular, SCF is produced in both soluble and membrane-bound forms (Flanagan *et al*, 1990b; Anderson *et al*, 1990), and the transmembrane form is missing in the Sl/Sl^{d} mutant (Flanagan *et al*, 1990b). Hence, transmembrane SCF is essential for normal haemopoiesis and, importantly, would not be detected in the conditioned medium of LTBMCs.

Biologically-active membrane-bound forms of other haemopoietic growth factors, for example IL-1 and M-CSF, have also been observed (Kurt-Jones *et al*, 1985; Stein *et al*, 1990). Furthermore, stromal cell-derived glycosaminoglycans in the bone marrow ECM are capable of binding both GM-CSF and IL-3 and presenting these factors in biologically active form to haemopoietic cells (Gordon *et al*, 1987; Roberts *et al*, 1988). As stromal cell-derived glycosaminoglycans appear to play a major role in the adhesion of primitive haemopoietic cells to the stroma (Aizawa *et al*, 1988; Tavassoli *et al*, 1990; Patel *et al*, 1989), and intimate contact between haemopoietic progenitors and stroma is required for prolonged haemopoiesis to occur in LTBMCs (Bentley, 1981; Verfaiille, 1992), this suggests: a) that positioning of haemopoietic progenitors close to membrane- or ECM-attatched haemopoietic growth factors might allow stimulation of haemopoiesis in certain areas of bone marrow with locally high concentrations of particular haemopoietic growth factors (Quesenberry, 1992); b) detection of a particular haemopoietic growth factor in LTBMCs may be difficult either because it is present but ECM- or membrane-bound (Flanagan *et al*, 1990b; Kurt-Jones *et al*, 1985; Stein *et al*, 1990; Gordon *et al*, 1987; Roberts *et al*, 1988), or because its gene regulation in stromal- or haemopoietic cells may be locally affected by intimate contact between stromal- and haemopoietic cells, so that the factor might only be produced by cells in such intimate contact.

Nevertheless, constitutive production by stromal cells of M-CSF, GM-CSF, G-CSF IL-6 and IL-3 mRNA has recently been reported, the latter only being detectable by PCR (Kittler *et al*, 1992). Further, factor-dependent haemopoietic progenitor cell-lines are supported by the above stromal cells, and this support is prevented by the addition of anti-IL-3, anti-GM-CSF, or anti-IL-6 neutralizing

antibodies (Kittler *et al*, 1992). This observation confirms that even subliminal levels of haemopoietic growth factors such as IL-3 are biologically active. In keeping with this, increasing the number of haemopoietic growth factors used in *in-vitro* stem cell assays allows the level of at least some of these factors to be reduced to subliminal levels (Quesenberry, 1992) with no reduction in effectiveness in rapid induction of proliferation of primitive haemopoietic stem cells (Quesenberry, 1992; Ikebuchi *et al*, 1988a; Ikebuchi *et al*, 1988b; Spangrude *et al*, 1988; Nakahata *et al*, 1982; Leary *et al*, 1989; Heimfeld *et al*, 1991).

The above suggests that "pockets" of haemopoiesis may exist in the bone marrow, where haemopoietic growth factors and stem cells are juxtaposed on the stromal ECM. However, it is also apparent from this discussion that multiple haemopoietic growth factors are potentially present throughout the bone marrow at concentrations that would be theoretically be sufficient in an *in-vitro* assay to allow proliferation of primitive haemopoietic stem cells, though in fact >90% of the stem cell compartment is quiescent in normal BM (Burgess *et al*, 1983). This dichotomy might be partially explained by the "niche" hypothesis (Schofield, 1979), whereby primitive haemopoietic stem cells are proposed to be in "niches", formed by stromal cells in the bone marrow, and therefore protected from haemopoietic growth factors until they are ejected from the "niche" and enter the proliferative and differentiative pathway (Schofield, 1979). However, as even primitive stem cells are rapidly proliferating in regenerating BM (Harrison *et al*, 1991), in which large amounts of a stimulator(s) (probably multiple upregulated haemopoietic growth factors) of stem cell proliferation are present (Lord *et al*, 1977), the primitive cells must not be entirely protected in "niches" in this situation. Furthermore, the more mature a member of the stem cell compartment, the less stimulus is required to stimulate proliferation (Hodgson *et al*, 1982; Lerner *et al*, 1990; Heimfeld *et al*, 1991), hence these cells would be even more prone to rapid proliferation when not required, due to exposure to growth factors required for the expansion of the progenitor compartment in normal haemopoiesis. These conceptual problems may be be potentially solved by the presence in normal BM of negative regulators of the proliferation of members of the stem cell compartment.

1.6 NEGATIVE REGULATORS OF STEM CELL PROLIFERATION.

1.6.1 Manchester Inhibitor:

An inhibitor(s) of *in-vitro* haemopoietic stem cell (CFU-S) proliferation is contained in normal BM and in the supernatant from confluent LTBMCs (Toksoz *et al*, 1980; Lord *et al*, 1976). The inhibition is reversible, non-toxic and specific for the stem cell (CFU-Sd12/CFU-A) compartment (Lord *et al*, 1976; Wright *et al*, 1977). The inhibitor appears to act by holding CFU-S cells at the G1/S boundary of the cell-cycle (Lord *et al*, 1979). Partial characterisation of the inhibitor showed it has a MWt of 50-100 kDa (Wright *et al*, 1979) and is produced by a sub-population of bone marrow macrophages distinct from that producing the stimulator of CFU-S proliferation (Wright *et al*, 1982; Wright *et al*, 1980). The macrophage-like cells *from normal BM* that in regenerating BM produce stimulator, and those from *regenerating BM* that in normal BM produce inhibitor, do not produce stimulator or inhibitor respectively when freshly-isolated (Lord *et al*, 1982), although the tissue carries the capacity for producing both. Complete separation of these two distinct sub-populations of bone marrow macrophages and incubation *in-vitro* allows low-level detection of the missing stimulator or inhibitor, respectively (Lord *et al*, 1982). It was therefore proposed that CFU-S proliferation in the bone marrow is governed by relative levels of stimulator and inhibitor, and that the CFU-S compartment somehow signals its numbers to the inhibitor-producing cells (Lord *et al*, 1982); depletion of the CFU-S compartment reduces this signal, so inhibitor production is reduced and relieves the block on stimulator production by the relevant cells. The localised ratios of CFU-S number to inhibitor- and stimulator-producing cells, rather than absolute CFU-S numbers, is deemed important in this model (Lord *et al*, 1982). Notably, CFU-Sd12 are much more sensitive to inhibitor than stimulator, while CFU-Sd8 are much more sensitive to the stimulator (Wright *et al*, 1979).

1.6.2 Transforming growth factor-beta.

Transforming growth factor beta (TGFβ) is a 25 kDa disulphide-linked homo-dimeric protein, which is thus far unique among cytokines in being secreted as a precursor that cannot bind its receptor until activated by proteolytic cleavage (Keller *et al*, 1992). A major source of TGFβ is the bone marrow (Keller *et al*, 1992), and it is readily-detectable in LTBMC conditioned media (Eaves *et al*, 1991).

Multiple non-allelic forms of TGFβ exist, each of which have similar effects on haemopoietic cells (differences are quantitative, not qualitative) (Keller *et al*, 1992). The effects of TGFβ, as for MIP1α (see below) are bi-directional:

1) *In-vitro*, TGFβ inhibits the formation of CFU-A colonies (Maltman *et al*, 1993), and also of more primitive members of the haemopoietic stem cell compartment (Keller *et al*, 1990), and only a 25-fold excess of a combination of *multiple* haemopoietic growth factors can overcome this negative effect (Bradley *et al*, 1991). TGFβ down-modulates the number of cell-surface receptors for the haemopoietic growth factors on the responding haemopoietic progenitor cells (Jacobsen *et al*, 1991), supporting the notion that relative levels of opposing positive and negative signals control stem cell proliferation (Lord *et al*, 1982).

TGFβ is readily detectable in the supernatant of LTBMCs (Eaves *et al*, 1991), and addition of anti-TGFβ antibody can prolong- or reactivate- primitive haemopoietic progenitor proliferation when added to previously stimulated- or quiescent- LTBMCs, respectively (Eaves *et al*, 1991). This suggests that the inhibitory activity detected in confluent LTBMCs (Toksoz *et al*, 1980) and hence perhaps that detected in normal BM (Lord *et al*, 1976) may at least in part be due to TGFβ.

2) In the presence of GM-CSF, TGFβ promotes a 3-5-fold increase in the number and size of bone marrow CFU-GM colonies, due to increased numbers of mature granulocytes (Keller *et al*, 1991). TGFβ and GM-CSF appear to act together to stimulate the growth of a novel progenitor cell, termed BFU-G (blast forming unit-granulocyte) (Keller *et al*, 1991). Further, TGFβ up-regulates the number of GM-CSF receptors on positively-responding bone marrow progenitor cells (Jacobsen *et al*, 1991b).

1.6.3 Tetrapeptide.

The tetrapeptide is the active component of a dialysable extract from foetal calf bone marrow, that prevents quiescent CFU-S from entering the cell-cycle (Lenfant *et al*, 1989). It is inactive on more mature haemopoietic progenitor cells and on proliferating CFU-S (Lauret *et al*, 1989; Monpezat *et al*, 1989). Although no *in-vitro* assay for the molecule has been developed, injection of neutralizing antisera to the tetrapeptide into normal mice results in the stimulation of endogenous CFU-S

proliferation, suggesting that the tetrapeptide may play a role *in-vivo* in the maintenance of CFU-S in the quiescent state (Frindel *et al*, 1989). The tetrapeptide *is* synthesized *in-vivo*, is present in NBM, and is synthesized and released by bone marrow cells in LTBMCs (Wdzieczak-Bakala *et al*, 1990).

Of the only three proteins that have been shown to contain the tetrapeptide sequence (Lenfant *et al*, 1989), thymosin-β4 is inducible by GM-CSF (Moscinski *et al*, 1990), suggesting the tetrapeptide is a feedback inhibitor of haemopoietic stem cell proliferation. An enzyme (Asp-N) that is able to cleave thymosin-β4 to produce the tetrapeptide has been identified (Grillon *et al*, 1990), and incubation of labelled thymosin-β4 with intact bone marrow cells results in the appearance of labelled tetrapeptide, suggesting that enzymatic cleavage of extracellular thymosin-β4 may occur in the bone marrow *in-vivo* (Grillon *et al*, 1990; Lenfant *et al*, 1991).

1.6.4 Haemoregulatory Peptide.

This molecule, initially identified in extracts of rodent bone marrow and human leukocytes, is a pentapeptide that can exist as a monomer or disulphide-bonded dimer (Laerum *et al*, 1984a; Laerum *et al*, 1984b). The monomer is inhibitory *in-vitro* to formation of GM-CFC-derived colonies, and *in-vivo* reduces GM-CFC and CFU-S numbers and prevents the recruitment of CFU-S into cycle following cytotoxic drug treatment (Laerum *et al*, 1984a; Laerum *et al*, 1984b; Paukowitz *et al*, 1990). The inhibitory effect of the haemoregulatory peptide (HP) on proliferation of the progenitor compartment appears to be restricted to cells of the myeloid lineages (Laerum *et al*, 1990). Interestingly, the dimer has no inhibitory activity, and is a potent stimulator of GM-CFC colony formation (Laerum *et al*, 1988), suggesting dimer/monomer ratio as a possible factor in determining myeloid progenitor cell levels and proliferation. Bone marrow, but not thymus- or spleen cells have been demonstrated to display receptors for the haemoregulatory peptide (Pauowitz *et al*, 1987).

1.7 STEM CELL INHIBITOR (SCI)/ MACROPHAGE INFLAMMATORY PROTEIN-1 ALPHA (MIP1α).

1.7.1 Involvement of MIP1α in the regulation of haemopoiesis:

The *in-vitro* CFU-A assay (Pragnell *et al*, 1988; Eckmann *et al*, 1988) was used to purify an 8 kDa protein termed stem cell inhibitor (SCI) from the conditioned

medium of a macrophage cell-line J774.2 (Graham *et al*, 1990). SCI consists of two proteins, both of which are identical to previously described proteins, MIP1α and MIP1β respectively (Davetalis *et al*, 1988; Sherry *et al*, 1989), which are the two components of a major heparin binding protein complex called MIP-1 secreted by endotoxin stimulated murine macrophages (Wolpe *et al*, 1988; Wolpe *et al*, 1989; Sherry *et al*, 1989; Davetalis *et al*, 1988). MIP1α is the main component active in inhibition of haemopoietic stem cell proliferation (Graham *et al*, 1990; Graham *et al*, 1993). Both recombinant MIP1α and purified MIP-1 form high molecular weight complexes of 10^5-10^6 Da as assessed by gel-filtration (Graham *et al*, 1990; Wolpe *et al*, 1988; Graham *et al*, 1993), raising the possibility that MIP1α and MIP1β exist in such a complex *in-vivo*, though recent data suggests that MIPα is only biologically active in a monomeric form (Broxmeyer *et al*, 1993).

SCI is antigenically indistinguishable from Manchester Inhibitor (Graham *et al*, 1990) and, like Manchester Inhibitor, reversibly inhibits both CFU-S and CFU-A proliferation *in vitro*, has no effect on proliferation of CFU-GM (Graham *et al*, 1990; Tejero *et al*, 1984), and appears to be more inhibitory to more primitive members of the stem cell compartment compared to more mature cells such as CFU-Sd8 (Wright *et al*, 1979; Dunlop *et al*, 1992). Hence, MIP1α and Manchester Inhibitor are probably identical. Additionally, a single *in-vivo* innoculum of MIP1α reduces the high proliferative levels of CFU-S observed in regenerating bone marrow to levels comparable to those seen in normal bone marrow (Dunlop *et al*, 1992), though it is claimed that MIP1α has no effect on the proliferation of long term repopulating stem cells *in-vivo* (Quesniaux *et al*, 1993).

Like TGFβ, MIP1α appears to have both inhibitory and stimulatory effects on different immature haemopoietic cell populations, as it is able to enhance CFU-GM colony formation (Broxmeyer *et al*, 1989; Broxmeyer *et al*, 1990). Both MIP1b and the MIP-1 complex also have this activity (Broxmeyer *et al*, 1989; Broxmeyer *et al*, 1990). Unlike TGFβ, MIP1α is not secreted as an inactive precursor molecule. However, MIP1β can bind to the same cell-surface receptor as MIP1α (Neote *et al*, 1993; Graham *et al*, 1993), and a 4:1 molar ratio of MIP1β to MIP1α, is able to block the inhibitory action of MIP1α on *in-vitro* colony formation by primitive members of the haemopoietic progenitor compartment (Broxmeyer *et al*, 1991). A 1:1

MIP1β:MIP1α molar ratio does not have this effect, in agreement with the ability of the MIP-1 complex to act as inhibitor of haemopoietic stem cell proliferation (Graham *et al*, 1990; Broxmeyer *et al*, 1991).

The antagonistic effect of MIP1β on MIP1α activity does not depend on continuous presence of MIP1β, as short exposure of bone marrow cells to- then removal of- MIP1β before the addition of MIP1α abrogates the effects of MIP1α in these assays (Broxmeyer *et al*, 1991). MIP1β has similar effects to MIP1α in terms of enhancement of *in-vitro* colony formation by haemopoietic progenitor cells (Broxmeyer *et al*, 1990), so the antagonistic effect of MIP1β appears to be relevant only to the inhibitory effects of MIP1α on more primitive haemopoietic cells. A recent report suggests that MIP1β has a stem cell inhibitory role at high concentrations (Graham *et al*, 1993); this apparent contradiction may possibly be explained by the different source of the MIP1α and MIP1β used by different investigators; for example, murine MIP1β has a potential glycosylation site (Graham *et al*, 1990; Broxmeyer *et al*, 1991; Graham *et al*, 1993).

1.7.2 Involvement of the MIP-1 proteins in the inflammatory response:

The MIP-1 proteins have other roles in addition to those on the haemopoiesis, though because MIP1α and MIP1β were originally co-purified, several observations were made on the functions of the MIP-1 complex, rather than on MIP1α and MIP1β separately. Thus, MIP-1 was shown to be mildly chemotactic for neutrophils *in-vitro* (Wolpe *et al*, 1988) (though this effect is not reproducible: Pragnell et al., personal communication), and injection of MIP-1 into the footpads of mice resulted in a localised inflammatory reaction, characterised by infiltration of neutrophils (Wolpe *et al*, 1988). Injection of MIP-1 into rabbits caused a rapid influx of neutrophils, followed by infiltration of monocytes (Saukkonen *et al*, 1990). MIP-1 is also the only known endogenous pyrogen that acts via a prostaglandin-independent pathway (Davetalis *et al*, 1989), and the MIP-1 complex modulates macrophage function, as it stimulates proliferation of mature tissue macrophages, and stimulates macrophages to release TNF-α, IL-6 and IL-1α (Fahey *et al*, 1992); co-stimulation with GM-CSF, M-CSF or IFN-γ, enhanced the latter effects of MIP-1 (Fahey *et al*, 1992).

No study on the individual efficacy of MIP1α or MIP1β to act as an endogenous pyrogen, or to act as a chemoattractant for neutrophils or monocytes *in-*

vivo has been published, though recent work suggests that MIP1α and MIP1β are chemotactic for monocytes but not neutrophils *in-vitro* (Schall, 1991) . MIP1α has recently been shown to activate both basophils and mast cells (Alam *et al*, 1992). Equivalent modulations of macrophage functions to that achieved by MIP-1 are achieved by MIP1α, but not by MIP1β (Fahey *et al*, 1992).

In fact, as little as 8-fold excess of MIP1β over MIP1α blocked significantly the macrophage TNFα induction by MIP1α (Fahey *et al*, 1992), analogously to the antagonistic effect of MIP1β on MIP1α inhibition of stem cell proliferation (Broxmeyer *et al*, 1991). MIP1α and MIP1β appear to be co-regulated in most expressing cell-types (Schall, 1991), though there is one report of differential expression of the two genes in myeloid progenitor cells (Schall, 1991). Given the antagonistic effects so far demonstrated for MIP1β on the functions of MIP1α, it will be necessary to investigate the relative expression of MIP1β in all MIP1α expressing cells and tissues.

1.7.3 MIP1α as a pan-stem cell inhibitor.

Purified (>95%), though not recombinant, MIP1α inhibits the proliferation of epidermal keratinocytes *in-vitro* (Parkinson *et al*, 1993). This is in contrast to the ability of both purified and recombinant MIP1α to function as inhibitors of haemopoietic stem cell proliferation (Graham *et al*, 1990), suggesting that an as yet unknown "accessory factor" is required in the keratinocyte system (Parkinson *et al*, 1993). The only cell-type so far found to express MIP1α in the skin are Langerhans cells (Matsue *et al*, 1992), which are related to tissue macrophages (Dexter *et al*, 1987), which themselves are believed to be a source of MIP1α in the bone marrow (Wright *et al*, 1980; Graham *et al*, 1990). This suggests that MIP1α produced by different tissue-macrophages may be a pan-stem cell inhibitor.

1.7.4 Human MIP-1 proteins:

Human homologues of both MIP1α and MIP1β exist (see below), though in contrast to the murine system, neither hMIP1β nor hMIP1α have the potential to be glycosylated (Graham *et al*, 1993). Both hMIP1α (LD78) and hMIP1β (Act-2) are effective *in-vitro* and *in-vivo* in the inhibition of haemopoietic stem cell proliferation (Broxmeyer *et al*, 1990; Graham *et al*, 1993), although hMIP1β, though much more

effective than muMIP1β, is less effective than human- or murine- MIP1α (Graham *et al*, 1993).

Both hMIP1α and hMIP1β may be important in lymphocyte trafficking, as they appear to target and attract separate sub-populations of lymphocytes (Schall, 1991). T cell chemotaxis and adhesion to endothelial cells is induced *in-vitro* by proteoglycan-immobilized hMIP1β (though apparently not by MIP1α) (Tanaka *et al*, 1993). hMIP1β and hMIP1α activate basophils *in-vitro* (Kuna et al, 1993), and hMIP1α, though not hMIP1β, induces the *in-vitro* migration and activation of normal human eosinophil granulocytes (Rot *et al*, 1992).

It is apparent from the above that investigation of the functions of murine- and human MIP1α and MIP1β are incomplete, though the available evidence suggests that the murine and human homologues of these molecules have similar if not identical functional activity.

1.7.5 Cell-restricted expression of MIP1α.

The cDNA sequence of MIP1α (Davetalis *et al*, 1988) reveals that it is the murine homologue of a cDNA termed pLD78 isolated from human tonsillar lymphocytes induced with TPA (an activator of protein kinase C, see chapter **4**) and PHA (a T cell mitogen) (Obaru *et al*, 1986). Several groups have now isolated cDNA and/or genomic sequences for human or murine MIP1α homologues. Using probes derived from such sequences, murine MIP1α mRNA has been detected in mast cells (Burd *et al*, 1989), activated T cells (Kwon *et al*, 1989a; Brown *et al*, 1989; Kwon *et al*, 1989b), macrophages (Davetalis *et al*, 1988) and Langerhans cells (Matsue *et al*, 1992). mRNA encoding human homologues for murine MIP1α has been detected in activated T cells (Obaru *et al*, 1986; Yamamura *et al*, 1989; Zipfel *et al*, 1989) and B cells (Obaru *et al*, 1986; Yamamura *et al*, 1989), bone marrow mononuclear cells (Nakao *et al*, 1992), peripheral blood monocytes (Yamamura *et al*, 1989; Blum *et al*, 1990) and myeloid progenitors (Nakao *et al*, 1990). In most of these cell types, the murine/human MIP1α transcript is at very low levels or is undetectable prior to the appropriate stimulation. Exceptions are fresh human tumour cells of monocytic-, T- and B-lymphocytic origins (Yamamura *et al*, 1989). Notably, low basal levels of murine MIP1α mRNA are detectable in macrophage cell-lines (Davetalis *et al*, 1988) and bone marrow derived macrophages (BMDMs) (A.Reid, PhD thesis (1992):

Glasgow University). It is evident from this data that both murine and human MIP1α gene expression may be confined to cells of haemopoietic origin. However, as this area of research is relatively new, this confinement of expression to haemopoietic lineages may be misleading. Hence, the isolated report of detection of human MIP1α transcripts in a human glioma cell-line and in primary human fibroblasts in response to PMA (an activator of protein kinase C, see chapter 4), may be significant (Nakao *et al*, 1990).

1.7.6 The possible roles of MIP1α *in-vivo*.

Like several other haemopoietic growth factors, MIP1α appears to be biologically active on stem, progenitor and mature haemopoietic cell compartments. Further, like these factors, MIP1α mRNA is present at only low levels in unstimulated macrophages, mast cells and Langerhans cells, and is barely- or un-detectable in other unstimulated expressing cell-types, and is highly and rapidly inducible, from which it might be inferred that MIP1α is important only in inducible situations. However, MIP1α protein and mRNA are detectable in normal BM (Wright *et al*, 1977; Wright *et al*, 1980; Graham *et al*, 1990; G.Graham 1993: personal communication). Further, as MIP1α is a heparin binding protein, and heparin and heparan sulphate are very similar glycosaminoglycans, this suggests that MIP1α secreted at low levels in normal BM might be sequestered in a biologically active form on stromal cells or stromal cell-derived ECM, as has been suggested for heparan sulphate-binding haemopoietic growth factors such as IL-3 and GM-CSF (Gordon *et al*, 1987; Roberts *et al*, 1988). This is consistent with the observation that regulation of stem cell proliferation occurs at a local level in the bone marrow (Wright *et al*, 1977), and with the "niche" hypothesis (Schofield, 1979) of stem cell regulation. The possible consequences of this "stickiness" of the MIP-1 proteins is well illustrated by the induction of T-cell adhesion to endothelial cells by proteoglycan-immobilized hMIP1β (Tanaka *et al*, 1993).

The concentration of MIP1α or MIP-1 required for stem cell inhibitory activity is in the picomolar range, while micromolar concentrations are required to provoke a response in assays of inflammatory function (Graham *et al*, 1990; Graham *et al*, 1993). Hence, like other regulators of haemopoiesis, low concentrations of MIP1α in the bone marrow might be important in the regulation of normal

haemopoiesis, correlating with the readily-detectable basal levels of MIP1α mRNA and protein in macrophages (Davetalis *et al*, 1988; Graham *et al*, 1990), which are the apparent source of MIP1α in the bone marrow (Wright *et al*, 1979; Wright *et al*, 1980). The much higher, induced levels of MIP1α, important in inflammatory situations, would be confined to the local area of inflammation by the heparin binding nature of MIP1α.

1.7.7 Cloning of the MIP1α gene.

Genomic clones for both murine and human MIP1α homologues have been obtained (Obaru *et al*, 1986; Blum *et al*, 1990; Nakao *et al*, 1990; Grove *et al*, 1990; Widmer *et al*, 1991). MIP1α appears to be a single-copy gene in mice (Widmer *et al*, 1991), but there are at least three functional human MIP1α genes, pLD78 (LD78β/GOS-19-2), LD78 and GOS-19-1 (Obaru *et al*, 1986; Blum *et al*, 1990; Nakao *et al*, 1990). The LD78β gene contains an Alu sequence in its promoter, but is still functional (Blum *et al*, 1990; Nakao *et al*, 1990). I was the first to clone the murine MIP1α gene (Grove *et al*, 1990 and see results section), so the detailed structure of the murine MIP1α gene and its homology to the human MIP1α genes is presented in the Results section.

1.8 THE PLATELET FACTOR FOUR (PF4) SUPERFAMILY.

1.8.1 The PF4 Family is Bipartite.

Murine MIP1α is 57% identical at the cDNA level and 60% identical at the amino acid level to murine MIP1β (Sherry *et al*, 1989). It is now apparent that MIP1β is merely the most highly related to MIP1α, of an expanding superfamily of related cytokine molecules (Schall, 1991). The members of this family, termed the PF4 superfamily, are related by sequence and by the conservation of a four-cysteine motif (Wolpe *et al*, 1989; Schall, 1991; Stoeckle, *et al*, 1990). The family is bipartite: in the "C-C" branch, the first two cysteines, situated towards the amino-terminal of the protein, are directly adjacent, while in the "C-x-C" branch, they are separated by a single amino acid. It has been shown for β-thromboglobulin, PF-4 and IL-8 of the C-x-C branch, and for MCP-1 of the C-C branch, that disulphide bridges link the first cysteine to the third and the second cysteine to the fourth, resulting in a secondary structure with two loops (Schall, 1991). Although all members of the superfamily

may have this secondary structure, there is no cross-competition between cell-surface binding sites for members of the C-C and C-x-C branches (Leonard *et al*, 1990; Samanta *et al*, 1989; Yoshimura *et al*, 1990). This correlates with the observations that human and murine C-C branch members are chemotactic for monocytes but not neutrophils (Scall, 1991; Leonard *et al*, 1990; Matsushima *et al*, 1989), and bind to, and transmit an intra-cellular signal from, the same cell-surface receptor (Neote *et al*, 1993; Graham *et al*, 1993), while C-x-C branch members are chemotactic for neutrophils but not monocytes (Matsushima *et al*, 1989; Baggiolini *et al*, 1989; Walz *et al*, 1992; Yoshimura *et al*, 1987) and share a different cell surface receptor (LaRosa *et al*, 1992). Although different members of the same sub-branch share the above functions, they also have unique functions (eg.see MIP1α, above), which may correlate with the detection of unique and shared receptors for members of the C-x-C sub-branch (LaRosa *et al*, 1992).

1.8.2 The C-C (RANTES/SIS) branch:

MIP1α is a member of the C-C branch of the PF4 superfamily (Schall, 1991). At present, five murine- and six human members (not including non-allelic versions of genes encoding identical proteins) of the C-C branch are known. These are (murine/human respectively):- MIP1α/hMIP1α (Davetalis *et al*, 1988; Obaru *et al*, 1986), MIP1β/hMIP1β (Sherry *et al*, 1988; Lipes *et al*, 1988), TCA-3/I-309 (Burd *et al*, 1987; Miller *et al*, 1989), JE/MCP-1 (Rollins *et al*, 1988; Yoshimura *et al*, 1989), C10/- (Orlofsky *et al*, 1991), -/RANTES (Schall *et al*, 1988), -/HC14 (Chang *et al*, 1989). In addition, an alternatively-spliced form of TCA-3, P500 has been cloned (Brown *et al*, 1989).

The C-C branch has alternatively been termed the RANTES/SIS (Regulated on Activation, Normal T Expressed and Secreted/ Small Inducible Secreted) family, as the proteins of this family are all small, of the order of 8-10 KDa, mostly induced from very low or undetectable levels in unstimulated cells to up to 1% of total polyA$^+$ RNA of the producing cells and secreted (Schall, 1991), though both RANTES and C10 mRNAs are present at high levels in unstimulated T cells and are markedly reduced in stimulated T cells (Schall, 1991; Orlofsky *et al*, 1991). Hence, most of these genes were originally cloned as cDNAs, on the basis of differential screening of cDNA libraries from resting compared to stimulated cells. Although the expression of

several members of the RANTES/SIS gene family, like the MIP-1 genes, may be restricted to cell-types of haemopoietic origin (Schall, 1991), this is not a general characteristic of this gene family, as RANTES and JE/MCP-1 are expressed in a wide range of cell-types (Schall, 1991; Rathanaswami *et al*, 1993; Kameyoshi *et al*, 1992; Heeger *et al*, 1992).

Each RANTES/SIS gene has a similar structure, with three exons and two introns, in which each exon encodes the equivalent section of each protein (eg. exon 1 encodes the 5' UTR and signal peptide in each case). Further, the position of the second intron in each gene is precisely maintained, and intron/exon junctions are highly-conserved between the different RANTES/SIS family members (Schall, 1991). The splice junctions are also conserved between RANTES/SIS and C-x-C families, though the C-x-C family genes contain a different number of exons and introns (Schall, 1991). The chromosomal position of all of the RANTES/SIS genes (except C10) have been mapped, and they map to the same locus, 11q11-11q21 in the mouse (Wilson *et al*, 1990), and 17q11-17q21 in the human (Irving *et al*, 1990; Miller *et al*, 1990; Donlon *et al*, 1989). Further, two of the human MIP1α and MIP1β genes are only 14kbp apart at this locus (Irving *et al*, 1990), in a head-to-head arrangement. The close linkage of these genes at this locus, plus a similarly close linkage of C-x-C family members on human chromosome 4 (Stoeckle *et al*, 1990) suggests an ancestral recombination event, followed by multiple duplications and divergence at each locus.

1.8.3 RANTES/SIS genes as "early-response" genes: the significance (or not) of a gene being an "early-response" gene.

A feature of the RANTES/SIS genes is that they are inducible (Schall, 1991). For example, murine or human MIP1α mRNA is highly inducible in under four hours in macrophages (Davetalis *et al*, 1988; Kwon *et al*, 1989), mast cells (Burd *et al*, 1989), T cells (Yamamura *et al*, 1989; Zipfel *et al*, 1989), B cells (Yamamura *et al*, 1989) and HL60 and U937 human myeloid progenitor cell lines (Yamamura *et al*, 1989).

JE was identified as a mRNA which is rapidly induced by treatment of quiescent murine 3T3 fibroblasts with platelet-derived growth factor (PDGF), which is a "competence" factor for 3T3 cell proliferation (Cochran *et al*, 1983). PDGF induction of JE is rapid (< 1 hour) and does not require protein synthesis, hence it is

induced as a primary response to the growth factor (Cochran *et al*, 1983; Rollins, 1991), and is an "early-response" or "primary-response" gene (Cochran *et al*, 1983; Rollins, 1991).

Because JE induction by PDGF, coincides with the commitment of the 3T3 cells to enter the cell-cycle, JE was considered to be a possible "competence gene", ie. required in order for the cells to pass from the G0 stage into the G1 stage of the cell-cycle (Cochran *et al*, 1983). Many investigators have used this technique of stimulating quiescent 3T3 fibroblasts with serum or purified serum growth factors such as PDGF, in an attempt to identify such "competence" genes (Cochran *et al*, 1983; Lan *et al*, 1985; Linzer *et al*, 1983). For example the proto-oncogene c-fos (Gillespie, 1991) (see below), is required for both exit from G0 and cell-cycle progression of 3T3 cells , as shown by anti-sense- and antibody micro-injection studies (Holt *et al*, 1986; Nishikura *et al*, 1987; Riabowol *et al*, 1988).

Several studies now suggest that c-fos may have several additional functions other than in the G_0-G_1 phase transition. Thus, mitogenesis can be dissociated from c-fos activation (Welham *et al*, 1990), c-fos is inducible to an equivalent level at all stages of the cell-cycle of 3T3 cells (Cortner *et al*, 1991), transient c-fos induction occurs in response to mitogenic stimuli, differentiation-inducing signals (Curran, 1988), and upon growth-factor withdrawal from factor-dependent cell-lines, therein being implicated in the initiation of the programmed cell-death pathway (Curran, 1988; Colotta *et al*, 1992). Further, c-fos is constitutively-expressed in some mature cell-types (Paterne *et al*, 1992), and is *not* required for the growth of most cell-types (Johnson *et al*, 1992; Field *et al*, 1992), but is involved in the development and function of several distinct tissues (Johnson *et al*, 1992; Field *et al*, 1992). Hence, though the early-response gene c-fos is required for 3T3 cells to enter the cell-cycle, it is clearly not required for this purpose in many cell-types, and has many other roles.

A primary objection to the involvement of RANTES/SIS gene products in cell-cycle progression of their cell of origin is that they are secreted cytokines (Schall, 1991). Secondly, these genes are upregulated in macrophages and/or fibroblasts by *cytostatic* agents such as LPS and IFN-γ (Davetalis *et al*, 1988; Tannenbaum *et al*, 1990; Yu *et al*, 1990; Martin *et al*, 1991), and glucocorticoids, which potentiate the mitogenic effect of serum growth factors on quiescent 3T3 cells, inhibit the induction

of JE mRNA (at the transcriptional level) by serum growth factors (Rameh *et al*, 1992). Hence, induction of RANTES/SIS genes does not necessarily correlate with entry into the cell-cycle, and in fact, although JE was originally termed a "competence" gene (Cochran *et al*, 1983), like c-fos, JE is equally inducible throughout the cell-cycle (Cortner *et al*, 1991).

Hence, early-response genes are not necessarily required for some fundamental role in the cell-cycle. The reason that JE is an early-response gene may be found, for example, in its role as a monocyte chemoattractant (Rollins *et al*, 1991). The early response to insults such as wounding or inflammation includes the attraction of leukocytes to the site of injury, and rapid production of JE or other members of the RANTES/SIS cytokine family at the site of injury would be expected to be involved in this response.

Other than JE, the early-response nature of members of the RANTES/SIS cytokine family has been little-studied. However, the basic criterion for being an early-response gene is satisfied by other members of this family. Thus, MIP1α is rapidly inducible (Davetalis *et al*, 1988; Burd *et al*, 1989; Kwon *et al*, 1989; Yamamura *et al*, 1989; Zipfel *et al*, 1989), and this induction occurs even in the presence of protein synthesis inhibitors (Zipfel *et al*, 1988; Nakao *et al*, 1990). Further the 3'UTR of the MIP1α gene contains repeated AU-rich sequences (Davetalis *et al*, 1988). These sequences are conserved in multiple cytokine and early-response genes (Caput *et al*, 1986) and are implicated in mRNA stability and translational control (Han *et al*, 1990; Shaw *et al*, 1986). The molecular basis of the function of such sequences will be discussed in chapter **4**, section **4.6**.

CHAPTER 2: TRANSCRIPTION FACTORS.

2.1 WHAT ARE TRANSCRIPTION FACTORS ?

Transcription factors are low-abundance non-histone proteins that bind to *cis*-acting gene-regulatory elements in a sequence-specific manner, and in so doing modulate the rate of transcriptional initiation of the gene concerned (Mitchell *et al*, 1989).

Transcription factors generally bind to DNA (Mitchell *et al*, 1989; Pabo *et al*, 1992), many of them in a dimerization-dependent manner (Pabo *et al*, 1992), where they interact with other transciption factors bound nearby or at a distance on the DNA, or with the transcriptional preinitiation complex (PC), to modulate transcriptional initiation (Lamb *et al*, 1991; Ptaschne, 1988; Gill *et al*, 1992, plus see chapter **3**). Hence, many transcription factors possess the ability to bind to DNA, and to form protein-protein interactions.

2.2 MODULAR STRUCTURE OF TRANSCRIPTION FACTORS.

Transcription factors are *modular*, in that different functions of a transcription factor are specified by relatively independent strucural domains (Brent *et al*, 1985; Frankel *et al*, 1991). Thus, the DNA binding domain of LexA, a bacterial repressor protein, fused to the transcriptional activation domain of a yeast transcription factor GAL4, leads to activation of GAL4-responsive genes in a LexA DNA binding site-dependent fashion (Brent *et al*, 1985). Further, in the case of the estrogen and glucocorticoid receptors, which are ligand-dependent transcription factors, modules can be interchanged to switch DNA binding, ligand binding and activation functions (Frankel *et al*, 1991). Because the positioning of the described modules is highly flexible, this suggests that each module represents an *independent* structural domain (Frankel *et al*, 1991). While this may be true in some cases, in others it is apparent that considerable interaction between domains within a transcription factor *do* occur. For example, the N- and C- terminal domains of the p110 precursor of the p50 NF-κB subunit have been shown to directly interact (Grimm *et al*, 1993). Transcription factors can be grouped into classes that use related domains, or domains that have common features.

2.3 DNA BINDING DOMAINS.

2.3.1 Overview.

Structural studies and sequence comparisons have led to the recognition of a growing number of classes into which transcription factors can be grouped according to the particular structural motif those factors use in order to bind DNA (Pabo *et al*, 1992; Johnson *et al*, 1989).

Many specific DNA binding proteins interact with DNA via a "recognition" α-helix, which is able to fit into the major groove of the DNA double-helix (Schwabe *et al*, 1991). Such α-helices appear to be widely used in this respect because they are intrinsically structurally stable when exposed on the surface of a protein structure (Schwabe *et al*, 1991).

The evolution of DNA binding proteins has resulted in the development of several different ways of presenting the DNA recognition helix to the DNA, by packing it against different stable structures (Schwabe *et al*, 1991). This stable presentation allows the exposed face of the recognition α-helix to bind to the major groove by making specific interactions with the bases and DNA backbone (Pabo *et al*, 1992; Schwabe *et al*, 1991).

2.3.2 Homeodomain:

The homeodomain, a DNA binding motif found in several Drosophila transcription factors, contains a structure called the helix-turn-helix (HTH) motif, which is common to many bacterial DNA binding proteins (Pabo *et al*, 1992; Johnson *et al*, 1989), and which consists of two α-helices separated by a turn (Pabo *et al*, 1992; Johnson *et al*, 1989). However, unlike isolated HTH units, the 60 amino acid homeodomain forms a stable structure and can bind DNA independently (Pabo *et al*, 1992). A typical homeodomain is that of the Engrailed protein of Drosophila, which consists of an extended N-terminal arm followed by three α-helices (Pabo *et al*, 1992). Helix 3 has one hydrophobic face, which packs against helices 1 and 2. The main DNA contacts are made by the exposed face of helix 3, the recognition helix, which fits directly into the major groove of DNA, and by the N-terminal arm, which fits into the minor groove and supplements the contacts made by helix 3 (Pabo *et al*, 1992), while the region containing helices 1 and 2 makes only two critical contacts with the DNA backbone (Pabo *et al*, 1992).

The HTH motif of the Drosophila homeodomain proteins (helices 2 and 3, above) contacts DNA in a way markedly different from that used by bacterial HTH-containing motifs (Pabo *et al*, 1992), which suggests that this particular structural motif has been adapted throughout evolution. A further adaptation in higher eukaryotes is that many homeodomain proteins contain amino acid sequence motifs flanking the homeodomain, that are conserved within specific sub-families (Pabo *et*

al, 1992), and which affect DNA binding. For example, the POU-domain class of transcription factors, which include transcription factors Oct-1, Oct-2 and Pit-1 (Rosenfeld, 1991), share an extensive 150 aa conserved POU domain, which contains two major regions of very high homology: the POU-specific (POU_S) domain (containing two highly conserved α-helices), and POU-homeodomain (POU_{HD}) (containing the classic three conserved homeodomain α-helices), which are separated by a poorly-conserved spacer region of about 20 aa's (Rosenfeld, 1991). Both the POU_S and POU_{HD} regions contact DNA and are required for DNA binding; high-affinity DNA binding is conferred by the POU_S domain and by basic regions at the amino terminus of the POU_S domain and flanking the POU_{HD} (Rosenfeld, 1991). The sequence-specificity of binding is conferred in POU-domain proteins by the POU_S domain (Rosenfeld, 1991).

2.3.3 "Classic" zinc finger:

This motif, first discovered in the Xenopus transcription factor TFIIIA, is widespread in eukaryotic transcription factors (Pabo *et al*, 1992; Johnson *et al*, 1989; Schwabe *et al*, 1991). Proteins in this family generally contain tandem repeats of the 30 aa zinc-finger motif, which consists of the sequence Cys-X_{N1}-Cys-X_{12}-His-X_{N2}-His , wherein N1=2-4 aa's, N2=3-5 aa's (Pabo *et al*, 1992; Johnson *et al*, 1989; Schwabe *et al*, 1991). Each zinc-finger forms an independently-folded domain, wherein a single zinc ion is tetrahedrally co-ordinated to the 2 cysteine and 2 histidine residues, resulting in the formation of a compact globular domain in which the central twelve amino acids form a loop or "finger" (Pabo *et al*, 1992; Johnson *et al*, 1989; Schwabe *et al*, 1991; Lee *et al*, 1989). The zinc-finger motif contains a β-sheet, conserved hydrophobic residues, and an α-helix (Pabo *et al*, 1992; Johnson *et al*, 1989; Schwabe *et al*, 1991; Lee *et al*, 1989). Specific contacts with the major groove are made by residues in the α-helix, and by an arginine that immediately precedes the α-helix (Pabo *et al*, 1992). Hence, the zinc-finger is a means of stabilizing the recognition α-helix, by holding it against a rigid β-sheet via a central zinc ion and a mini-hydrophobic core, so that the recognition helix can fit into the major groove.

2.3.4 Steroid Hormone Receptor Zinc-Finger:

Steroid hormone receptor (SHR) zinc-fingers are superficially similar to classic zinc-fingers, in that finger formation involves tetrahedral co-ordination of a

central zinc ion (Schwabe *et al*, 1991). The SHR DNA binding domain contains two zinc fingers, wherein the central zinc ions are tetrahedrally co-ordinated to 4 cysteine- (rather than 2 cysteine- and 2 histidine-) residues (Schwabe *et al*, 1991).

However, the SHR zinc-fingers contain no conserved hydrophobic residues, and are more widely separated than classical zinc-fingers (Schwabe *et al*, 1991), and although α-helices are present, these extend from the C-termini of each finger, rather than being part of the fingers (Schwabe *et al*, 1991). Notably, conserved hydrophobic residues are present *between* the two fingers.

In contrast to the classical zinc-finger, the two Zn^{2+}-binding motifs of SHRs fold together to form a single structural domain, rather than folding independently (Schwabe *et al*, 1991). The first helix, which contains conserved basic residues, is believed to be the DNA recognition helix, and is packed against the second helix, forming an extensive hydrophobic core with a zinc ion forming the amino terminus of each helix (Schwabe *et al*, 1991).

The second finger also forms protein-protein interactions between SHR monomers, influencing the spacing and relative orientation of the two receptor monomers, which aligns their recognition helices, allowing DNA contact in two adjacent major grooves of the DNA (hence, the typical palindromic DNA site recognized by SHRs) (Schwabe *et al*, 1991).

2.3.5 bZIP transcription factors.

A large number of eukaryotic transcription factors contain a structure called the leucine zipper, which consists of a heptad repeat of leucines over a region of 30-40 residues with, generally, a conserved repeat of hydrophobic residues occurring 3 residues to the N-terminal side of the leucines (Pabo *et al*, 1992; Alber, 1992). The leucine zipper forms a dimerization interface, in which a parallel 2-stranded α-helical coiled-coil is formed, whereby the conserved hydrophobic residues (ie. leucines plus the other conserved hydrophobic residues) are buried in the interface of the coiled-coil dimer (Pabo *et al*, 1992; Alber *et al*, 1992), forming a continuous hydrophobic interface.

The basic region adjoining the leucine zipper (LZ) is about 30 residues long, and is primarily responsible for the sequence-specificity of DNA binding by LZ proteins (Pabo *et al*, 1992; Alber *et al*, 1992). This region appears to be only about

25% α-helical until DNA binding, upon which it is 100% α-helical (Pabo *et al*, 1992; Alber *et al*, 1992).

The basic region-leucine zipper (bZIP) domain appears to adopt a Y-shaped structure, in which the parallel coiled-coils of the LZ region form the stem of the Y, and the basic regions form the α-helical arms of the Y, which extends in oposite directions along the major groove of the DNA, making contact with about 10 bp of DNA (Pabo *et al*, 1992; Alber *et al*, 1992). There are several families of bZIP proteins, various of which can form homo- and heterodimers intra- and inter-family (see chapter **4**, section **4.5.2**). Negative regulators of bZIP proteins also exist, that can dimerise, but which have non-functional basic regions (see chapter **4**, section **4.5.2**).

2.3.6 bHLH transcription factors.

A second large class of tissue-specific and ubiquitous transcription factor proteins, for which dimerisation is also a prerequisite for DNA binding, also contain a basic DNA binding region adjacent to a dimerization motif (Pabo *et al*, 1992; Kadesch *et al*, 1992; Edmonson *et al*, 1992). The dimerization motif consists of two conserved amphipathic α-helices, separated by an intervening unstructured loop (Pabo *et al*, 1992; Kadesch *et al*, 1992; Edmonson *et al*, 1992), hence these proteins are called basic-helix-loop-helix (bHLH) proteins.

Recent models suggest that bHLH transcription factor DNA binding occurs via the interaction of hydrophobic faces of the helices, and formation of a 4-helix bundle in which the helices are parallel (Edmonson *et al*, 1992; Vinson *et al*, 1992). It is suggested that the basic region extends from helix 1 as an α-helix, allowing interaction of conserved residues of the basic region with the major groove, and sequence-specific DNA binding (Vinson *et al*, 1992).

All transcription factors that contain the bHLH domain recognize a conserved core DNA motif called the E-box (Edmonson *et al*, 1992; Vinson *et al*, 1992; Visvader *et al*, 1991). Members of the bHLH family bind this motif as homo- or heterodimers, though heterodimers appear to bind significantly better to E-box-containing sites than do homodimers (Edmonson *et al*, 1992). As with the bZIP class of proteins, certain proteins exist that dimerize with bHLH proteins but contain non-functional basic regions, so binding to the E-box is prevented (Benezra *et al*, 1990).

2.3.7 bHLH-Zip transcription factors.

A large group of transcription factors contain bHLH motifs, bind to E-box DNA sequences, by a mechanism proposed to be the same as that used by bHLH proteins (Vinson *et al*, 1992), but do not dimerize with the class of bHLH proteins described above. This second group of bHLH-containing proteins contain one or more LZ motifs, similar to those of the bZIP proteins, in addition to the bHLH domain (Vinson *et al*, 1992; Fisher *et al*, 1991; Beackmann *et al*, 1991). The LZ in bHLH-LZ proteins is generally located immediately carboxy-terminal to helix 2 of the HLH motif (Benezra *et al*, 1990; Fisher *et al*, 1991; Beckmann *et al*, 1991; Hu *et al*, 1990; Davis *et al*, 1993; Reddy *et al*, 1992), and models predict that helix 2 and the LZ form an extended α-helix (Vinson *et al*, 1992; Beckmann *et al*, 1991). The LZ of several bHLH-LZ proteins has been observed to stabilize dimer formation and in some cases is absolutely required for dimer formation (Beckmann *et al*, 1991; Hu *et al*, 1990), which may be due to the relatively poor hydrophobic character of individual LZ and helix (of the HLH motif) motifs (Beckmann *et al*, 1991).

Unlike members of the bHLH class of proteins, bHLH-LZ proteins do not promiscuously heterodimerize, as they only heterodimerize with a limited subset of bHLH-LZ proteins (Fisher *et al*, 1991; Beckmann *et al*, 1991; Hu *et al*, 1990; Davis *et al*, 1993; Reddy *et al*, 1992). This limited heterodimerization may be because HLH helix 2 and the LZ are differently spaced in different proteins, so that in the extended α-helix that they form, they would be separated by different integral numbers of α-helical turns (Vinson *et al*, 1992; Beckmann *et al*, 1991). Further, certain LZs are incompatible, ie. they do not form a coiled-coil (Beckmann *et al*, 1991). In at least some cases, the HLH motif also contributes to the specificity (compatibility) of dimerization (Davis *et al*, 1993). Interestingly, c-Fos, a member of the bZIP family of proteins, has recently been shown to interact- and regulate transcription in co-operation with a bHLH-ZIP protein called FIP (Blanar *et al*, 1992).

2.3.8 Helix-turn-helix-like.

Certain eukaryotic transcription factors contain DNA binding domains which are reminiscent of the helix-turn-helix motif first identified in the DNA binding domains of bacterial repressor proteins, and later as part of the Drosophila transcription factor homeodomain. These include Myb-family members (Frampton *et*

al, 1989), and members of the IRF-family of proteins, that bind to interferon-alpha-responsive elements in gene regulatory regions (Veals *et al*, 1992).

2.3.9 ETS domain.

An expanding family of transcription factors contain homology within an 85 residue region that confers DNA binding and is termed the ETS-domain (Karim *et al*, 1990; Nye *et al*, 1992). These transcription factors recognize *cis*-acting sequences containing a GGA core motif (Karim *et al*, 1990; Nye *et al*, 1992). The ETS domain contains a highly basic region in the carboxy-terminal half of the domain and a highly-conserved N-terminal 10 residue region that is leucine-rich (Karim *et al*, 1990; Nye *et al*, 1992). ETS domain proteins can bind to DNA in monomeric form, or in association with other proteins (Karim *et al*, 1990; Nye *et al*, 1992).

2.3.10 Rel Homology domain.

A family of transcription factors including c-Rel, and components of the NF-κB transcription factor contain a 300 residue region of homology that is required for DNA binding (Grimm *et al*, 1993). Hence, in comparison with more well-characterized DNA binding domains, the Rel homology domain (RHD) is unusual, if only for its length (Grimm *et al*, 1993). Dimerization by members of this family is a prerequisite for DNA binding and, like bZIP and bHLH proteins, the region that specifies dimerization is contiguous with the DNA binding domain (the C-terminal half of the RHD contains the region required for dimerization) (Grimm *et al*, 1993).

2.4 PROTEIN-PROTEIN INTERACTION DOMAINS CONTAINED BY TRANSCRIPTION FACTORS.

2.4.1 Overview.

Certain transcription factor families require to dimerize via a common motif (contained on both of the interacting proteins), in order to bind to DNA. Transcription factors can also enter into protein-protein interactions, either within or between families, via like- or unlike motifs, with a variety of other functional outcomes. In many of these interactions, the DNA binding domain of the transcription factor involved mediates such interactions.

2.4.2 Involvement of transcription factor DNA binding domains in protein-protein interactions:

2.4.2.1 POU domain proteins:

POU-domain proteins can bind as monomers to their cognate DNA recognition elements (Rosenfeld, 1991). However, in keeping with the fact that many of their DNA binding sites contain direct repeats, DNA-dependent homodimers have been observed to form via cooperative binding interactions, conferred by the POU domain, and resulting in increased transcriptional activity (Rosenfeld, 1991).

The POU domain also mediates DNA-independent heterodimer formation between different family members, resulting in increased DNA binding (Rosenfeld, 1991), while a factor called I-POU has been observed to specifically interact with the POU domain of certain POU-domain proteins, preventing DNA binding (Rosenfeld, 1991). In contrast to this, a viral co-activator protein, VP16, which contains a strong transcriptional activation domain (see later) but cannot bind to DNA alone, interacts specifically with the POU_{HD} of Oct-1, which promotes interaction of VP16 with DNA and confers a strong transcriptional activation potential on Oct-1 (Rosenfeld, 1991; Thompson *et al*, 1992).

2.4.2.2 bHLH proteins.

Certain bHLH proteins are expressed-, and function-, tissue-specifically (Edmonson *et al*, 1992), for example MyoD protein is involved in muscle-specific transcriptional activation (Edmonson *et al*, 1992). Domain-swop experiments with non-muscle-specific bHLH transcription factors revealed that the basic region of these other factors did not change the dimerization or DNA binding activity of the MyoD protein, but resulted in chimeric proteins that could not activate muscle-specific genes (Edmonson *et al*, 1992). Two residues of the approximately 30 residue basic region are conserved *only* in myogenic bHLH proteins (Edmonson *et al*, 1992), and may confer interaction of these muscle-specific bHLH proteins with co-activator protein(s) that mediate muscle-specificity (Edmonson *et al*, 1992).

2.4.2.3 LZ-containing transcription factors.

In addition to the leucines and other hydrophobic residues conserved in the LZ dimerization motif of bZIP and bHLH-LZ proteins, certain residues on the solvent-exposed face of LZs are conserved between, for example, c-Myc (bHLH-LZ protein) and C/EBPα (bZIP protein) (Lamb *et al*, 1991). This led to the hypothesis that these conserved residues could mediate interaction of these proteins with other proteins,

possibly a shared partner (Lamb *et al*, 1991). In keeping with this, the LZ of the heterodimer bZIP proteins Fos-Jun interacts with the zinc fingers of glucocorticoid receptor (Lamb *et al*, 1991) (see later section for functional consequences). Further, the LZ of the C/EBPβ protein (bZIP protein) mediates protein-protein interactions between CEBPβ and the Rel Homology domain of the Rel family protein p50 (LeClair *et al*, 1992).

2.4.2.4 RHD transcription factors.

In addition to mediating dimerization between Rel-family proteins (Grimm *et al*, 1993) and interaction between bZIP and Rel-family proteins (LeClair *et al*, 1992), the RHD mediates intra- and inter-protein-protein interactions between Rel-family proteins and a family of proteins called the I_KB proteins (Grimm *et al*, 1993), via repeats of a 33 residue motif of the I_KB proteins, called the **ankyrin repeat** (Hoffman, 1992; Grimm *et al*, 1993). Such interactions have a variety of functional consequences, as discussed in chapter **4**, section **4.5.1**.

2.4.2.5 ETS domain transcription factors.

Ankyrin repeats also play a role in enhancing the DNA binding activity of an ETS family protein, GABPα (Hoffman, 1992; Thompson *et al*, 1991), which binds weakly as a monomer to DNA, via its ETS domain (Thompson *et al*, 1991). However, the ETS domain, plus an adjacent 37 residue region, facilitates DNA-independent protein-protein interaction with the ankyrin repeat domain of a protein called GABPβ (Hoffman, 1992; Thompson *et al*, 1991). A dimer of GABPβ forms a tetramer with two GABPα molecules, facilitating strong binding of GABPα to two GGA-containing hexamer repeats, and DNA contact of GABPβ molecules adjacent to these repeats (Hoffman, 1992; Thompson *et al*, 1991).

The DNA binding of several other ETS domain proteins has either been implied or shown to be enhanced by their association with other unrelated proteins (Nye *et al*, 1992), including the Ets-1 protein and the Serum Response Element (SRE) associated proteins SAP-1 and Elk-1 (Nye *et al*, 1992, and see chapter **3**, section **3.3.4**). In addition, binding of the macrophage- and B-cell-specific ETS-domain protein PU.1 to its cognate element allows PU.1 to recruit a second nuclear factor to an adjacent *cis*-acting site on the DNA (Nye *et al*, 1992). The specific involvement of the ETS domain in these interactions has not as yet been demonstrated.

2.4.3 Involvement of transcription factor activation domains in protein-protein interactions:

Many transcription factors possess one or more transcriptional activation domains, which are domains that promote protein-protein interactions, resulting in stabilization/enhancement of binding of the transcriptional preinitiation complex (PC) at the transcriptional start site, thus increasing the rate of transcriptional initiation (Mitchell *et al*, 1989; Ptaschne *et al*, 1990; Ptashne, 1988).

The proteins contacted by activation domains can be: a) components of the preinitiation complex (PC) (Stringer *et al*, 1990; Lin *et al*, 1991; Ing *et al*, 1992; Liao *et al*, 1991); b) other transcription factors, either heterologous or homologous, by which action a potentially better surface of interaction with components of the PC is formed (Su *et al*, 1991; Pascal *et al*, 1991; Li *et al*, 1991); c) factors tightly associated with the PC (Gill *et al*, 1992; Tanese *et al*, 1991; Pugh *et al*, 1990). Further, certain transcription factors which do not contain transcriptional activation domains, can associate with *co-activators*, which contain activation domains themselves, but cannot associate with DNA in the absence of the correct DNA-bound transcription factor (Thompson *et al*, 1992; Forsberg *et al*, 1991).

Several types of transcriptional activation domain have so far been characterized. These include *acidic* activation domains, which are characterized by their significant negative charge, sequence flexibility and lack of structure (Mitchell *et al*, 1989; Sigler *et al*, 1988), *glutamine-rich* activation domains, which characteristically contain very few charged amino acids (Mitchell *et al*, 1989), and *proline-rich* activation domains (Mitchell *et al*, 1988 *et al*, Mermod *et al*, 1989). Certain activation domains are a combination of the above (Mermod *et al*, 1989), while the proline-rich activation domain of transcription factor CTF-1 contains several stretches of serine- and threonine residues (Mermod *et al*, 1989), which could be phosphorylated or glycosylated (allowing or preventing, respectively, the addition of negative charge. The mechanisms by which activation domains work will be discussed in more detail in chapter 3, section **3.6.2.**

CHAPTER 3: GENE EXPRESSION.

3.1 OVERVIEW.

In-vitro and *in-vivo* functional studies of RNA Polymerase II transcribed gene regulation have generally involved transfection into cells of chimaeric constructs, consisting of the coding sequence of a reporter gene, linked downstream of the particular DNA sequence being tested for gene-regulatory function (Serfling *et al*, 1985; Dynan *et al*, 1985). These studies led to the identification of several types of *cis*-acting gene regulatory elements, which all bind transcription factors in a modular fashion, though differing in distance from the site of transcriptional initiation and the number of transcription factors that they bind.

3.2 BASAL PROMOTER.

This is situated immediately 5' to the RNA Polymerase II transcriptional initiation site (CAP site). Basal promoters can be generally classified as either containing or not containing a "TATA" box, which is the consensus binding site for a protein complex called TFIID (Salzman *et al*, 1989). In genes that contain a TATA box, transcription initiates 25-30 bp downstream from the TATA box (Smale *et al*, 1989).

Basal promoters for genes that do not contain an obvious TATA box can be divided into two classes: 1) GC-rich promoters, found primarily in housekeeping genes, that contain several transcription initiation sites spread over up to several hundred bps, and which contain multiple consensus binding sites for certain transcription factors such as Sp1 (Dynan *et al*, 1986; Pugh *et al*, 1991; Dynan *et al*, 1985); 2) Promoters, of genes that unlike housekeeping genes are not constitutively transcriptionally active, that are not GC-rich and contain no obvious TATA box, but which initiate transcription at only one or a few tightly clustered start sites (Smale *et al*, 1989). Such promoters contain a sequence called the "Initiator" (Inr), that spans the transcriptional start site (Smale *et al*, 1989). The Inr, which is also present in some TATA-containing promoters, weakly binds RNA Polymerase II (Caracamo *et al*, 1991; O'Shea-Greenfield *et al*, 1992). In the absence of a TATA box, the Inr appears to direct RNA Polymerase II to a precise transcriptional start site, while in the

presence of a TATA box, it may lead to stronger activity of a basal promoter (Caracamo *et al*, 1991; O'Shea-Greenfield *et al*, 1992).

Both "TATA" and non-"TATA" basal promoters require the TFIID complex for function (Pugh *et al*, 1991; Salzman *et al*, 1989; Caracamo *et al*, 1991). For example, Sp1 appears to tether the TFIID complex to GC-rich non-"TATA" basal promoters (Pugh *et al*, 1991). Binding of TFIID to the basal promoter allows the recruitment of RNA Polymerase II and other basal transcription factors required for transcription (Buratowski *et al*, 1989; Cortes *et al*, 1992).

TFIID is a multi-protein complex, containing TATA binding protein (TBP), and sixteen or more TBP-associated factors ("TAFs"). TAFs appear to mediate the effects of promoter- and enhancer binding transcription factors (see below) by interacting with these transcription factors in protein-protein interactions , allowing stabilization of the preinitiation complex (PC) at the transcriptional start site (Pugh *et al*, 1991; Sharp *et al*, 1992; Zhou *et al*, 1992; Gill *et al*, 1992; Ptaschne *et al*, 1990; Tanese *et al*, 1991; Pugh *et al*, 1990).

3.3 PROMOTER.

3.3.1 Overview.

The promoter element, immediately upstream of the basal promoter, is of the order of 50 to several hundred bps in length, and modulates the activity of the basal promoter (Barrera-Saldana *et al*, 1985; Nishizawa *et al*, 1990). As MIP1α is an early-response gene, the promoter of the early-response gene *c-fos* is used as an example of a "typical" promoter in the following discussion. As for many early-response gene promoters, the *c-fos* gene promoter directs a low level of basal transcription, which is rapidly and transiently induced by various cell stimuli (Hayes *et al*, 1987).

3.3.2 Modularity of promoters.

Promoters are *modular*, as they contain several functional domains, or modules, which by themselves have no or weak promoter activity, but which synergistically alter the rate of transcriptional initiation to a much greater extent (Jones *et al*, 1988). Thus, the *c-fos* gene promoter (human and murine) contains three modules, at ~-300 bps (distal), ~-170 bps (medial) and ~-60 bps (proximal) relative to the CAP site (Lucibello *et al*, 1991; Runkel *et al*, 1991; Hipskind *et al*, 1991), and

mutations in any of these modules alters basal and induced *c-fos* promoter activity (Runkel *et al*, 1991; Wagner *et al*, 1990; Treisman, 1992; Velcich *et al*, 1990; Shaw *et al*, 1989a; Karim *et al*, 1990; Shaw *et al*, 1989b; Treisman, 1990; Fisch *et al*, 1989a; Metz *et al*, 1991; Fisch *et al*, 1989b; Konig *et al*, 1989). Individual modules are not specific to particular promoters, and different promoters contain different combinations of various modules (Jones *et al*, 1988).

3.3.3 Modules bind multiple transcription factors.

Each module contains binding site(s) for tissue-specific and/or ubiquitous transcription factor(s) (Jones *et al*, 1988). Thus, the *c-fos* distal promoter module contains four transcription factor binding sites: a *sis* Inducible Factor (SIF) element, a Serum Response Element (SRE), an ETS-domain transcription factor binding site, and a Fos-AP-1-like (FAP) element (Hayes *et al*, 1987; Wagner *et al*, 1990; Treisman, 1992; Velcich *et al*, 1990; Karim *et al*, 1990; Hipskind *et al*, 1991; Treisman *et al*, 1992; Dalton *et al*, 1992). The SIF element and the ETS-domain transcription factor binding site bind, respectively, a single factor or different members of the same transcription factor family (Hayes *et al*, 1987; Wagner *et al*, 1990; Shaw *et al*, 1989b; Hipskind *et al*, 1991; Treisman *et al*, 1992; Dalton *et al*, 1992). The SRE can bind Serum Response Factor (SRF) (Treisman, 1990), a homeodomain protein called Phox-1 (Grueneberg *et al*, 1992), the bZIP protein C/EBPβ (Metz *et al*, 1991), two different zinc finger protein called YY1 and SRE-ZBP (Gualberto *et al*, 1992; Attar *et al*, 1992), and the bHLH protein E12 (Metz *et al*, 1991b), while the FAP element can bind AP-1 and C/EBPβ transcription factors (Velcich *et al*, 1990; Metz *et al*, 1991a).

3.3.4 Co-operative-, antagonistic- and cell-specific binding of transcription factors to promoter modules:

The DNA binding sites for different transcription factors within a promoter module may be either closely-abutting, thus allowing (or promoting) co-binding of the transcription factors to the promoter, or overlapping and therefore suggesting competition for DNA binding of transcription factors at that site. DNA binding (and competition for DNA binding) to such a site may be cell-specific and/or mediate the effects of different signals transduced from the cell surface.

In the *c-fos* gene distal promoter module, the SIF, ETS-, FAP and SRE sites are closely abutting. Induction of *c-fos* transcription by serum requires the SRF binding to the SRE and ETS-family proteins binding to the ETS-site (Treisman, 1992; Karim *et al*, 1990; Shaw *et al*, 1989; Treisman, 1990; Hipskind *et al*, 1991; Treisman *et al*, 1992; Dalton *et al*, 1992), and the latter cannot bind to the ETS-family recognition site in the absence of SRF bound at the SRE (Shaw *et al*, 1989). Similarly, though both Phox-1 and SRF can bind to the SRE, Phox-1 binds to SRF in the absence of DNA, and enhances SRF DNA binding, possibly by bringing SRF into the vicinity of the SRE. Further, the HLH protein E12 appears to bind to the SRE in association with SRF, and it is suggested that an SRF-E12 complex may be responsible for nerve growth factor induction of *c-fos* transcription via the SRE in PC12 cells (Metz *et al*, 1991b).

In addition to these co-operative and/or simultaneous DNA binding interactions, C/EBPβ binds to the SRE in PC12 cells in a cAMP-dependent mannner, displacing SRF in the process (Metz *et al*, 1991a; Metz *et al*, 1991b). Further, YY1 binding to the SRE represses serum-inducible and basal expression of the *c-fos* promoter (Gualberto *et al*, 1992), and SRE-ZBP binding to the SRE is also implicated in repression of the *c-fos* promoter.

3.3.5 Early-response gene promoters: Constitutive occupation by transcription factors of promoter modules, and use of the same promoter modules for basal-, induced and repressed transcription:

If promoter modules are unoccupied by transcription factors, nucleosomes bind at those sites, and DNA replication is generally required before transcription factors are again able to bind to the promoter (see chapter **5**, section **5.3.7**). As transcription of many early-response genes is rapidly and transiently induced within minutes in expressing cell-types, this means that transcription factors must continually occupy the promoter, even when transcription is low or non-existent, so that the promoter is "poised" for induction, and that the same promoter modules are used by transcription factors that modulate basal-, induced- or repressed transcription.

Investigation, using genomic footprinting, of *in-vivo* nucleoprotein binding to the *c-fos* promoter reveals that, upon epidermal growth factor (EGF) treatment of various cell-types, which induces *c-fos* transcription via elements including the SRE

and the FAP site (Herrera *et al*, 1989; Fisch *et al*, 1989; Shaw, 1992; Malik *et al*, 1991), the protein-DNA contacts made over the ETS-, SRE- and FAP sites are identical prior to and during induction-, and during subsequent repression of transcription (Herrera *et al*, 1989; Gille *et al*, 1992). Induction of *c-fos* transcription by EGF appears to involve specific phosphorylation of ETS-family protein(s) by MAP kinase, allowing increased ternary complex formation between ETS family proteins and SRF binding to the SRE (Gille *et al*, 1992; Malik *et al*, 1991). Hence, prior to the transient transcriptional induction, the *c-fos* promoter is "poised", and induction may involve post-translational modification and possibly rapid exchange of members of the same family of transcription factors binding at the same *c-fos* promoter site (Herrera *et al*, 1989; Gille *et al*, 1992). Further, prior to induction, SRF binding at the SRE appears be involved in repression of *c-fos* promoter activity (Shaw *et al*, 1989b; Shaw, 1992), so that three levels of *c-fos* expression, induced, repressed and constitutive, appear to involve occupation of the SRE by SRF and interaction of SRF with other proteins binding to the *c-fos* distal promoter module.

3.4 ENHANCERS.

Early studies on gene regulation revealed that sequences upstream of the SV40 "early" promoter stimulated transcription of a linked reporter gene in transfections by more than two orders of magnitude (Banerji *et al*, 1981; Moreau *et al*, 1981; Fromm *et al*, 1983), and this enhancement occurred when the element was placed in either orientation, and >3000 bps up- or downstream from the promoter and coding sequences of the reporter gene (Banerji *et al*, 1981; Moreau *et al*, 1981; Fromm *et al*, 1983). Enhancers are a general characteristic of viral and cellular systems, and are found in positions ranging from the proximal promoter region of a gene to many thousands of bps away from the transcriptional start site (Atchison *et al*, 1988; Evans *et al*, 1990).

Like promoters, enhancers are modular (Serfling *et al*, 1985; Atchison *et al*, 1988). The enhancer effect depends on the mixture of sequence motifs present, and also upon the presence or absence of particular transcription factor(s) that interact with that motif in a particular cell-type (Atchison *et al*, 1988).

The distinction between enhancer and promoter has become blurred, as various promoters have enhancer-like properties, and many enhancers can act as promoters (Atchison *et al*, 1988). Indeed, transcription factors that interact with promoters also interact with enhancers and vice-versa (Herbomel *et al*, 1990; Minie *et al*, 1992). For example, the erythroid cell transcription factor GATA-1 has binding sites in the promoters and enhancers of all the genes in the multi-gene family of the α- and β-globin gene clusters (Minie *et al*, 1992).

3.5 SILENCERS.

Certain gene-regulatory elements *repress* transcription in a position- and orientation-independent manner and, like enhancers, can modulate promoter activity when several thousand bps away from the transcriptional start site (Brand *et al*, 1985; Rivier *et al*, 1992). These elements are termed silencers (Brand *et al*, 1985; Rivier *et al*, 1992). Silencers associated with many higher-eukaryotic genes have been identified, acting in a developmentally-regulated and/or tissue-specific manner (Cao *et al*, 1989; Wada-Kijama *et al*, 1992). As silencers and enhancers are modular and mediate promoter activity in a position- and orientation-independent manner (Brand *et al*, 1985; Rivier *et al*, 1992; Cao *et al*, 1989; Wada-Kijama *et al*, 1992; Baniahmad *et al*, 1990), the molecular mechanism(s) behind silencer/enhancer activity is likely to be similar, as discussed below.

3.6 HOW DOES GENE REGULATION WORK ?

3.6.1 Communication between enhancers and promoters.

Various models have been proposed for the mechanism of action of enhancers (Ptaschne, 1986). In the *twisting* model, regulatory proteins binding at an enhancer are proposed to untwist the DNA, propagating the change in conformation up to the site of the (promoter and) transcriptional start site, hence allowing proteins such as TFIID and RNA Polymerase II to bind DNA (Ptaschne, 1986). However, experiments in which the enhancer is topologically separated from the gene, yet still has its effect argue against this idea (Ptaschne, 1986; Muller *et al*, 1990; Dunaway *et al*, 1989).

These experiments also argue against the *sliding* model, wherein a protein(s) is proposed to bind to a regulatory site such as a promoter or enhancer, then track

(slide) along the DNA to another specific sequence, where it allows initiation of transcription, perhaps by interacting with another protein(s) (Ptaschne, 1986). In spite of one recent example to the contrary (Herendeen *et al*, 1992), evidence suggests that tracking is a rare occurrence, and may not apply to higher eukaryotic gene regulation (Ptaschne, 1986; Herendeen *et al*, 1992).

In the "*oozing*" model, a protein binds to the gene regulatory sequence, allowing another to bind to it, etc., resulting in a processsion of proteins oozing out from the regulatory site to the site of transcriptional initiation (Ptaschne, 1986). Co-operative binding of proteins to regulatory sites *can* occur (see section **3.3.4** plus Ptaschne, 1986), as can the formation of transcription factor multimers at a regulatory site, via protein-protein interactions (Pascal *et al*, 1991); however, there is no evidence for the "oozing" of transcription factors over many thousands of bps.

Ptaschne et al. first showed that bacterial viral gene regulatory proteins bind co-operatively to DNA and interact by looping of the DNA between bound proteins (Ptaschne, 1986). Experiments using topologically-separated enhancer and promoter/basal promoter sites (Ptaschne, 1986; Muller *et al*, 1990; Dunaway *et al*, 1989) further reinforced the looping model, and DNA looping between promoter and enhancer sequences separated by 1.8 kbp mediated by activation domains of DNA-associated SP-1 transcription factors has been observed by electron microscopy (Su *et al*, 1991). Thus, most evidence suggests that communication between enhancers and promoters occurs by looping-out of the DNA between transcription factors bound at these sites.

3.6.2 Communication between promoter/enhancer-bound transcription factors and the preinitiation complex (PC).

Protein-protein interactions, involving transcription factor activation domains, promote the binding and stabilization of the preinitiation complex (PC) at the basal promoter, resulting in modulation of the rate of transcriptional initiation (Ptaschne, 1988). Various components of the PC have been demonstrated to be capable of directly or indirectly interacting with transcription factors (Gill *et al*, 1992). Transcription factors containing either proline- or glutamine-rich activation domains have been demonstrated to require TFIID-associated TAFs in order to activate transcription (Gill *et al*, 1992; Tanese *et al*, 1991; Pugh *et al*, 1990), while various

acidic activation domains have been shown to interact directly with TBP or to require TAFs in order to interact with TFIID (White *et al*, 1991; Flanagan *et al*, 1991; Stringer *et al*, 1990; Flanagan *et al*, 1990). The direct binding of viral acidic activation domains to TBP may reflect that these viral proteins have evolved to mimic cellular co-activators (Gill *et al*, 1992).

The basal transcription factor TFIIB has also been demonstrated to directly bind a viral acidic activating region, which correlates with the fact that assembly of TFIIB into the PC is a rate-limiting step which is enhanced by acidic activators (Lin *et al*, 1991). TFIIB also interacts directly with members of the steroid hormone receptor superfamily, independently of any co-activator(s) (Ing *et al*, 1992).

RNA Polymerase II itself has been implicated in the interaction between acidic transcriptional activators and the PC (Liao *et al*, 1991), via the carboxyl-terminal domain (CTD) of the largest subunit of RNA Pol II, which contains multiple tandem hepatapeptide repeats (Herbomel *et al*, 1990). However, it now appears that the CTD is involved in effecting the transition from transcriptional initiation to elongation (Lu *et al*, 1992), as it specifically interacts with TBP when in non-phosphorylated form, but cannot bind when phosphorylated (Usheva *et al*, 1992). Phosphorylation of the CTD is achieved by basal transcription factor TFIIH (Lu *et al*, 1992).

Hence, transcription factor activation domains can interact with at least two (possibly three) components of the PC. Further, as TFIID contains multiple TAFs, which results in TFIID being a very large complex of ~750 kDa which covers ~60 bps of promoter sequences (Zhou *et al*, 1992), there is a large area of the PC available for interaction with transcription factor activation domains. Hence, it is likely that multiple promoter/enhancer-bound transcription factors can interact simultaneously with the PC.

3.6.3 How transcription factors synergistically modulate the rate of transcriptional initiation.

The simultaneous binding of transcription factors to different components of the PC (ie.TFIIB and TFIID) should allow at least an additive effect on transcriptional initiation, as addition of TFIID and TFIIB are separate rate-limiting steps in the PC formation (Buratowski *et al*, 1989). Similarly, if multiple transcription factors

recognised the same site on, say, TFIID, increasing the number of transcription factors would be expected to have an additive effect on transcriptional initiation. However, a modular promoter or enhancer, which can bind several transcription factors at adjacent sites, allows TFIID (or other components of the PC) to "see" any one of these bound sites, and binding of TFIID to a factor at one site will present TFIID to a neighbouring site, promoting binding to TFIID of the factor bound there, etc. (Herbomel *et al*, 1990).

This model (Herbomel *et al*, 1990) forms the basis for the observed synergistic enhancement of transcription by multiple elements in a modular promoter or enhancer. There is no distinction between promoter and enhancer, other than that the promoter is next to the transcriptional start site and hence allows presentation of TFIID to its correct binding site on DNA (TFIID can recognize a range of apparently unrelated sequences (Wiley *et al*, 1992)).

3.6.4 Specificity of action of promoters/enhancers.

Some transcription factors are expressed in a cell-specific manner, for example GATA-1 is erythroid-, mast- and megakaryocyte cell-specific (Engel *et al*, 1992), and PU.1 is macrophage- and B-cell-specific (Klemz *et al*, 1990), while others, for example SRF, are more ubiquitously expressed (Treisman, 1990). However, where a transcription factor is expressed in a cell-restricted manner, it is often the case that other members of the same transcription factor family exist, which are capable of binding to exactly the same DNA sequence, and which are more widely expressed or expressed in a different set of tissues. GATA-2 and GATA-3 are members of the same family as GATA-1 and fit the above criteria (Engel *et al*, 1992), while several ETS family members can bind to the same DNA sequence as PU.1 and are more widely expressed (Thompson *et al*, 1992; Ray *et al*, 1992).

In the case of any particular tissue-specific promoter or enhancer, it is likely that factors will exist in a given non-expressing cell-type that are capable of binding to some if not all of the sites on that promoter/enhancer, and yet the tissue-specific gene is not expressed. One explanation for this, the *jigsaw hypothesis* is based on the fact that even if the same transcription factor binding sites are present in different promoters, each promoter has a distinct ordering of these sites, and only certain transcription factors that can bind each site will be capable of fitting into the "slot"

(ie. binding will not be hindered, and may be encouraged, by transcription factors binding to the adjacent sites) on the promoter between two adjacently-bound factors (Thompson *et al*, 1992; Johnson *et al*, 1989).

For example, a HSV immediate-early gene enhancer contains repeated CGGAAR motifs adjacent to a TAATGARAT motif (Thompson *et al*, 1992). The TAAT motif can bind members of the Oct family of transcription factors; however, of these factors, Oct-1 best binds the viral protein VP-16, which can then interact with the adjacent GARAT motif (Thompson *et al*, 1992). An additional cellular factor(s), HCF, binds to VP-16 and is necessary for the formation of the complex (Thompson *et al*, 1992). The adjacent CGAAR motif weakly binds an ETS-family protein, GABPα. This binding is greatly strengthened by interaction between GABPα and GABPβ, which also makes specific contact with the CGAAR motif (Thompson *et al*, 1992). Notably, no other ETS-domain protein apart from GABPα, appears to associate with GABPβ (Thompson *et al*, 1992).

3.6.5 Promoter specificity conferred by TAFs.

An extension of the jigsaw hypothesis involves non-DNA binding proteins: transcription factor-associated *co-activators* and TBP-associated TAFs. It has been suggested that promoter-specific TAF/TBP complexes may exist (Sharp, 1992; Pugh *et al*, 1991), such that different TFIID molecules would contain distinct TAF combinations, providing the complementary surface for the unique arrangement of sequence-specific transcriptional regulators bound to a particular promoter (Sharp, 1992; Pugh *et al*, 1991).

Although some investigators dispute this model, there is good evidence to support it. *Firstly*, the existence of TBP in complexes associated with different TAFs has been demonstrated (Rigby, 1993), interestingly as a component of TFIID and of the equivalent complexes that are essential for PC formation in RNA PolI and RNA Pol III transcribed genes (Rigby, 1993). *Secondly*, TBP is only 38 kDa in size yet can associate with at least 16 (maybe >20) different TAFs in forming TFIID (Pugh *et al*, 1991; Sharp, 1992; Zhou *et al*, 1992; Gill *et al*, 1992; Ptaschne *et al*, 1990; Tanese *et al*, 1991; Pugh *et al*, 1990), suggesting that all of these cannot bind simultaneously. In keeping with this, some TAFs appear to be present in sub-molar ratios in TFIID (Pugh *et al*, 1991). *Thirdly*, evidence suggests that transcription factors "select" the correct

TFIID molecule in order to activate transcription. For example, SRF activates SRE-dependent transcription *in-vitro* when it has been pre-incubated with highly-purified TFIID before addition to the rest of the PC components and the DNA. However, if the PC is allowed to assemble on the DNA before the addition of SRF, no SRE-dependent activation of transcription is observed (Zhu *et al*, 1991). One interpretation of this is that SRF can only interact with (select) a sub-population of multiple different TFIID molecules. "Squelching" experiments, wherein high levels of some transcription factors prevent activation by themselves or certain other activators (Ptaschne *et al*, 1990), may represent a similar mechanism. A corrollary of this discussion is that if the correct TFIID complex (ie.the correct TAFs) for a particular promoter is not available in a particular cell-type, that cell-type will not express the promoter in question.

Recombinant TBP can bind, more weakly than to TATA sites, to sites ~-30 bp upstream of the cap site (same position as the TATA box) of certain non-TATA promoters (Wiley *et al*, 1992). In these promoters, the context of the TBP binding site within the promoter may be important, so that association of TBP with different cellular factors may determine its affinity for a particular binding site (Wiley *et al*, 1992). A corrollary to this is that different TATA box sequences might be expected to bind different forms of TFIID. This is strongly supported by studies where the TATA box of the myoglobin gene is mutated to that of the SV40 promoter, resulting in loss of responsiveness to a muscle-specific enhancer (MSE) (Wefald *et al*, 1990). Conversely, incorporation of the myoglobin gene TATA box sequence into the SV40 promoter conferred responsiveness to the MSE (Wefald *et al*, 1990). The difference in the TFIID molecules binding to these different TATA boxes could be conformationional or because TBP is associated with different TAFs (Wefald *et al*, 1990). Interestingly, cell-specificity of expression is conferred by the particular sequence of the the TATA box of the PF4 cytokine gene (Ravid *et al*, 1991).

3.6.6 Promoter specificity conferred by transcription factor-associated co-activators:

In addition to possible cell-specific TAFs, cell-specific transcription factor co-activators may also be involved in the expression of cell-specific genes. For example, the SV40 enhancer enhances the activity of several minimal promoters in a variety of

cell-types; in contrast though an E2-dependent enhancer enhances the activity of these same minimal promoters in lymphoid cells, in other cell-types it is only effective in enhancing the activity of a subset of these promoters (Forsberg *et al*, 1991). Thus, the E2-dependent enhancer appears to require cell-type-specific co-activator(s) in order to communicate (presumably by looping) with transcription factors bound to the promoter.

A corrolary to this finding is that certain transcription factor activation domains may interact with other transcription factors, rather than with components of the PC. For example, the SP-1 transcription factor contains four activation domains (Pascal *et al*, 1991), some of which appear to be involved in interaction between SP-1 and TFIID-associated TAFs (Pugh *et al*, 1991), and in formation of DNA-bound SP-1 multimers, but one of which is essential for the formation of multimers of SP-1 at SP-1 DNA binding sites (Pugh *et al*, 1991). This multimerization is an essential step in the synergistic activation of transcription by two SP-1 binding sites (Pugh *et al*, 1991). SP-1 has also been demonstrated to directly interact with the E2 transcription factor (Li *et al*, 1991), and an E2-dependent enhancer activates transcription from an SP-1-bound minimal promoter (in contrast to other minimal promoters) in a non-cell-specific manner (Forsberg *et al*, 1991).

3.6.7 How silencers and negatively-acting transcription factors work.

It is evident that there are numerous points in the enhancer/promoter mediated regulation of transcription at which a silencer or negative regulator of transcription could work. For example, a factor(s) binding to a silencer could interact with a promoter-bound factor, in such a way as to prevent further interaction with the PC, or a promoter-bound negative regulator could lack a domain(s) allowing interaction with enhancer-bound proteins, or components of the PC. Also, it is well known that different members of transcription factor families contain activation domains of widely-differing activity, while certain transcription factor family members do not contain an activation domain (Chiu *et al*, 1989; Descombes *et al*, 1991; Grimm *et al*, 1993). Competitive binding of such factors having the same promoter/enhancer binding site, but with widely-differing activation domains could therefore be an important transcriptional regulatory mechanism.

Certain factors which can act as positive regulators of transcription when in different promoter context, achieve negative promoter regulation simply by binding at a site directly over/overlapping the PC basal promoter binding site. Examples of this are GR negative regulation of the IL-6 promoter (GR also occludes an IL-6 gene enhancer simultaneously) (Ray *et al*, 1990), p53 negative regulation of the *c-fos* promoter (Kley *et al*, 1992), and BPV-1 E2 repression of promoter activity (Dostatni *et al*, 1991) by binding over the site where the PC should form.

Finally, as TFIID-associated TAFs are involved in positive regulation of transcription, it might be expected that negatively-acting TAFs exist. In support of this idea, a 19 kDa phosphoprotein called Dr1, that binds to TBP and represses both basal and activated transcription , has been described (Inostroza *et al*, 1992). As for other TAFs, association of Dr1 with TBP promotes binding of TBP to the TATA box (Inostroza *et al*, 1992). However, association of unphosphorylated Dr1 with TBP plus basal transcription factor TFIIA, prevents basal transcription factor TFIIB from joining the complex, and phosphorylated Dr1 prevents association of TBP with *any* of the basal transcription factors (Inotroza *et al*, 1992).

CHAPTER 4: SIGNAL TRANSDUCTION.

4.1 OVERVIEW.

Although the regulatory regions of early-response genes are in a "poised" conformation prior to the appropriate cell stimulation, the cellular mRNA levels of such genes are very low or undetectable (see chapter **1**, section **1.8.3** and chapter **3**, section **3.3.5**). Upon the appropriate cell-stimulation, for example serum, there is a rapid and (generally) transient accumulation of the early-response genes' mRNA (Cochran *et al*, 1983; Rollins, 1991; Gillespie, 1991). In order for these changes to occur, rapid signalling must take place from the cell-surface, through the cytoplasm and into the nucleus. As the subject of this manuscript is transcriptional regulation, the mechanisms whereby cell-surface signalling modulates the rate of transcriptional initiation will be discussed in more detail, though other mechanisms by which the

production of early-response proteins is modulated in response to such signalling will be briefly discussed.

A model of a "typical" early-response signal transduction pathway is: Ligand binding to cell-surface receptor -> receptor protein tyrosine phosphorylation -> signalling protein tyrosine phosphorylation -> signalling protein serine-threonine phosphorylation -> transcription factor serine-threonine phosphorylation (= modulation of transcription factor activity) -> modulation of transcription of early-response gene. As with all such models, an ever-increasing number of exceptions are coming to light. Thus, while the activity of protein phophatases would be expected to be crucial for down-regulation of such a kinase-mediated cascade, it is now apparent that phosphatases play a positive role in at least some signal transduction pathways. Further, direct tyrosine phosphorylation of certain transcription factors, resulting in modulation of their activity, has been demonstrated.

The following discussion concerns examples of cell-surface receptor types, intracellular signalling molecules (protein kinases and phosphatases), and transcription factor targets of such signalling pathways, mediating early-response gene transcriptional modulation.

4.2 CELL-SURFACE RECEPTORS.

4.2.1 Overview.

Most extra-cellular ligands recognise specific cell-surface receptors (Jaye *et al*, 1992; Seuwen *et al*, 1992; Cadena *et al*, 1992; Taga *et al*, 1993; Bolen *et al*, 1992), which act as a transmembrane signalling system, through which extra-cellular ligands communicate with the cell nucleus to modulate gene expression (Cohen, 1992). There are several known types of cell-surface receptor, which transduce the signal from their particular extra-cellular ligands by different means. The receptor types relevant to a discussion of early-response genes are: i) the receptor tyrosine kinases (Jaye *et al*, 1992; Cadena *et al*, 1992), ii) the G-protein-coupled receptors (Seuwen *et al*, 1992) and iii) the cytokine receptors (Taga *et al*, 1993; Bolen *et al*, 1992). Various cytokine receptors can fall into either of the first two of these categories (Jaye *et al*, 1992; Taga *et al*, 1993; Bolen *et al*, 1992), though most do not.

4.2.2 Receptor Tyrosine Kinases.

All receptor tyrosine kinases (RTKs) share common features such as an extracellular region involved in ligand binding, and a cytoplasmic tyrosine kinase domain (Jaye *et al*, 1992; Cadena *et al*, 1992). Binding of ligand to the extracellular domain induces oligomerisation of receptors, resulting in increased interaction of cytoplasmic tyrosine kinase domains, and receptor *auto*phosphorylation by a *trans*-mechanism (Jaye *et al*, 1992; Cadena *et al*, 1992). Receptor autophosphorylation allows the association of intracellular substrates, or "second-messengers" with phosphotyrosine residues on the receptor cytpolasmic tail, via conserved motifs called SH2 domains, contained in such substrates (Jaye *et al*, 1992; Cadena *et al*, 1992). Different SH2 domains recognise different phosphotyrosines in different receptor cytoplasmic tails, such that different receptors are recognised by overlapping but distinct subsets of SH2 domain-containing substrates (Jaye *et al*, 1992; Cadena *et al*, 1992; Seuwen *et al*, 1992). Many RTK substrates themselves possess catalytic activity which is regulated by tyrosine phosphorylation. Important examples of such molecules are: phospholipase C-gamma (PLC-γ), Ras-GTPase activating protein (Ras-GAP), p85,the putative regulatory subunit of phosphatidylinositol 3' kinase (PI-3' kinase), and members of the c-Src family of non-receptor tyrosine kinases (Jaye *et al*, 1992; Pawson, 1992; Seuwen *et al*, 1992), all of which become tyrosine phosphorylated and activated upon binding to RTKs (Jaye *et al*, 1992; Pawson, 1992; Seuwen *et al*, 1992). For example, the association of PLC-γ with tyrosine-phosphorylated RTKs results in tyrosine phosphorylation and concommitant increased activity of PLC-γ (Seuwen *et al*, 1992; Posada *et al*, 1992). The substrate of PLC-γ is phosphatidylinositol 4,5 bisphosphate (PIP_2), which is membrane-associated (Seuwen *et al*, 1992; Posada *et al*, 1992; Asaoka *et al*, 1992), cleavage of which yields inositol triphosphate (IP_3) and diacylglycerol (DAG) (Seuwen *et al*, 1992; Posada *et al*, 1992; Asaoka *et al*, 1992). IP_3 triggers the release of stores of intracellular calcium, which is an activator of serine/threonine kinases called calmodulin-regulated kinases (Cam kinases) (Cohen, 1992; Seuwen *et al*, 1992; Posada *et al*, 1992), and, with DAG, a co-activator for serine/threonine kinases of the protein kinase C (PKC) family (Asaoka *et al*, 1992; Cohen, 1992; Seuwen *et al*, 1992; Posada *et al*, 1992). Hence, activation of PLC-γ by receptor tyrosine kinases results in the translation of that signal into the activation of protein serine/threonine kinases.

Ras-GAP, along with NF-1, is a GTPase, that promotes the hydrolysis of GTP to GDP by Ras, a small membrane-associated protein with intrinsic GTPase activity that is an *essential* component of many complicated receptor-mediated signal transduction pathways (Satoh *et al*, 1992; Downward, 1992; Hall, 1992). Ras can exist in a GDP- or a GTP-bound form, and it is only the GTP-bound form that can transmit signals (Satoh *et al*, 1992). The tyrosine phosphorylation of Ras-GAP mediated by RTKs leads to the association of Ras-GAP with two proteins, p62 and p190. The association of Ras-GAP with p190 which, in addition to other characteristics, appears to be a transcription factor, is speculated to involve Ras (Satoh *et al*, 1992; Downward, 1992; Hall, 1992). Thus, confusion exists over the precise role(s) played by GAPs such as Ras-GAP and NF-1 in Ras-mediated signal transduction, in that these proteins are implicated as both negative regulators and downstream effectors of activated Ras (Satoh *et al*, 1992; Downward, 1992; Hall, 1992). Further confusion over Ras-mediated signal transduction may in part be explained by the cell-specificity of such pathways. For example, in some cell-types PKC can activate Ras, while in other PKC is a downstream effector of Ras activity (Satoh *et al*, 1992), and in certain cell-types PI-3' kinase is essential for activation of Ras, whereas in others it is not required (Satoh *et al*, 1992). Nevertheless, Ras modulates the activity of several serine-threonine and threonine-tyrosine kinases involved in signal transduction cascades. Ras protein is required (at least in some cell-types) for the activation of Raf-1 (protein serine-threonine kinase), mitogen activated protein (MAP) kinases (also known as the *erk* family of protein serine-threonine kinases), MAP kinase-kinase (protein threonine-tyrosine kinase) and S6 kinase (a member of the rsk family of protein serine-threonine kinases) (Satoh *et al*, 1992). Recent evidence suggests that Raf-1 operates downstream of Ras, but upstream of MAP kinase-kinase, which is involved in the activation of MAP kinases, which in turn activate Rsk-family protein serine/threonine kinases (Satoh *et al*, 1992). Also, PKC appears to be required for activation of MAP kinase in some cell-types (Satoh *et al*, 1992).

PKC family members, Erk- and Rsk kinases are nuclearly- and cytoplasmically located, and the latter two translocate into the nucleus upon cell-stimulation (Chen *et al*, 1992; Greif *et al*, 1992), and are directly imlplicated in

serine-threonine phosphorylation of transcription factors or their associated molecules (Chen *et al*, 1992; Greif *et al*, 1992). Further, casein kinase II, a ubiquitous protein serine-threonine kinase found in the cytosol and nucleus also appears to be activated as an end-point in a Ras-mediated signal transduction pathway, and is involved in transcription factor phosphorylation (Seuwen *et al*, 1992; Gauthier-Rouviere *et al*, 1991).

4.2.3 The G-protein-coupled receptors.

Many cell-surface receptors involved in early-response gene activation are directly coupled to G-proteins, a family of small heterotrimeric GTP-binding proteins that are related to-, but functionally distinct from Ras proteins, and include those for members of the PF4 superfamily of cytokines (Seuwen *et al*, 1992; Taga *et al*, 1993).

Like Ras, G-proteins have inactive GDP-bound- and active GTP-bound forms (Seuwen *et al*, 1992). Binding of extracellular ligand to the receptor is believed to induce GDP to GTP exchange, and dissociation of the active α-subunit, which interacts with an effector enzyme, stimulating or inhibiting the enzyme's activity (Seuwen *et al*, 1992). The α-subunit has intrinsic GTP-ase activity, so is self-regulatory, and reassociates with the $\beta\gamma$ subunit upon deactivation (Seuwen *et al*, 1992). Effector enzymes relevant to early-response gene activation, that are activated by G proteins, include phospholipases, such as PLC (hence PKC and Cam kinases are activated by both RTKs that can interact with PLC-γ, and by G protein-coupled receptors (Cohen, 1992; Seuwen *et al*, 1992; Posada *et al*, 1992; Asaoka *et al*, 1992)) and PLA_2, and adenylate cyclase (Seuwen *et al*, 1992). Similarly, in certain cell-types, the Ras-mediated pathway of signal transduction could be expected to be activated via G protein-coupled receptors (Seuwen *et al*, 1992; Satoh *et al*, 1992). In contrast to PLC-γ, which appears to lead to transient activation of PKC, enzymes such as PLA_2 and PLD, an enzyme believed to be activated by by G protein-coupled receptors, break down phosphatidylcholine, generating activators that can lead to sustained activation of PKC (Seuwen *et al*, 1992; Asaoka *et al*, 1992). Adenylate cyclase mediates the generation of cAMP, a direct activator of the protein serine-threonine kinase protein kinase A (PKA) (Seuwen *et al*, 1992) and receives either stimulatory- or inhibitory signals from the appropriate receptor-associated G protein, G_S or G_I, respectively (Seuwen *et al*, 1992).

As for the serine/threonine kinases described above, activated PKA translocates to the nucleus upon activation, and is involved in direct and indirect modulation of the activities of different transcription factors involved in the transcriptional regulation of early-response genes (Seuwen *et al*, 1992; Hunter *et al*, 1992; de Groot *et al*, 1992). PKA may also modulate other signal transduction pathways, for example by phosphorylation of PLC-γ and hence inhibition of PIP_2 breakdown (Seuwen *et al*, 1992).

4.2.4 Cytokine receptors and other (haemopoietic cell) signal transducers.

Although some do-, many cytokine receptors and other hemopoietic cell surface signal transducers do not contain a cytoplasmic tyrosine kinase domain and are not coupled to G-proteins (Seuwen *et al*, 1992; Gauthier-Rouviere *et al*, 1991; Shaw *et al*, 1991). It is characteristic of this third class of receptors, that a receptor-associated molecule is required in order to achieve high affinity ligand binding and/or transmission of cytoplasmic signals (Taga *et al*, 1993). Many, if not all of these receptors mediate the transduction of the extra-cellular signal by means of association of non-receptor protein tyrosine kinases of the Src family with either the receptor or a receptor-associated protein (Taga *et al*, 1993; de Groot *et al*, 1992; Shaw *et al*, 1991). For example, Fyn binds to the T cell receptor (TCR) -associated ζ-subunit, and is implicated in signal transduction, while Lyn associates with the GM-CSF receptor and the IL-3 receptor, and Lck, Fyn and Lyn with the IL-2 receptor β-subunit (Bolen *et al*, 1992). Notably, dominant-negative Ras prevents signal transduction mediated by Src (Rim *et al*, 1992), and this correlates with the fact that cell stimulation with cytokines such as IL-3, GM-CSF and IL-2 results in up-regulation of the amount of cellular Ras-GTP, which is blocked by tyrosine kinase inhibitors (Taga *et al*, 1993; Satoh *et al*, 1992). Further, cell-stimulation by IL-2, IL-3 or GM-CSF leads to activation of c-Raf, which (see earlier) appears to be a downstream effector of Ras-mediated signal transduction. Hence, many cytokine- and hemopoietic cell-surface receptors may mediate early-response gene transcriptional modulation via similar pathways as those mediated by RTKs.

4.3 PHOSPHATASES IN SIGNAL TRANSDUCTION.

4.3.1 Overview.

The transient nature, especially on tyrosine, of many cellular protein phosphorylation events implies the involvement of protein phosphatases in cellular signal transduction pathways, as a means of attenuating the transduced signal (Fischer *et al*, 1991; Pallen *et al*, 1992). Both tyrosine- (receptor-like and non-receptor-like (Fischer *et al*, 1991; Pallen *et al*, 1992)) and serine/threonine- phosphatases exist, and the latter can be divided into four sub-classes, PP-1, PP-2A, PP-2B and PP2C, on the basis of substrate specificity and dependence on divalent cations for activity (Pallen *et al*, 1992; Cohen, 1992b).

4.3.2 Tyrosine phosphatases.

Receptor-like protein tyrosine phosphatases (PTPases) may play a positive role in the activation of signal transduction pathways. This is illustrated by CD45, an abundant hemopoietic cell-specific trans-membrane receptor-like PTPase (Bolen *et al*, 1992; Fischer *et al*, 1991; Pallen *et al*, 1992; Mustelin *et al*, 1993). T cell activation is achieved by signal tranduction involving the T cell receptor (TCR) complex, and appears to involve Fyn and Lck, members of the Src family of non-receptor protein tyrosine kinases (Bolen *et al*, 1992; Fischer *et al*, 1991; Pallen *et al*, 1992; Mustelin *et al*, 1993). Protein tyrosine kinases of the Src family are negatively regulated by interaction of their SH2 domain with an intra-molecular regulatory C-terminal phosphorylated tyrosine (Bolen *et al*, 1992). Current models suggest that activation of Fyn and Lck depends on a balance between dephosphorylation-, mediated by CD45, and phosphorylation of this regulatory tyrosine, mediated by a protein tyrosine kinase called $p50^{csk}$ (Bolen *et al*, 1992; Fischer *et al*, 1991; Pallen *et al*, 1992; Mustelin *et al*, 1993). In contrast to this positive role of CD45 in signal transduction, certain transmembrane PTPases appear to be involved in accelerated deactivation of receptor tyrosine kinase activity (Hashimoto *et al*, 1992).

In *Drosophila*, Corkscrew, a non-receptor PTPase, acts distal to a receptor tyrosine kinase and, in conjunction with Raf serine/threonine kinase, effects the downstream activation of two putative transcription factor-encoding genes (Pallen *et al*, 1992). The Corkscrew PTPase contains two SH2 homology regions, suggesting interaction with phosphotyrosine residues on its substrates (Pallen *et al*, 1992; Ahmad *et al*, 1993). Interestingly, two mammalian PTPases, both containing two SH2 domains, have also recently been identified (Ahmad *et al*, 1993). All three of these

PTPases contain putative C-terminal MAP kinase phosphorylation sites (Ahmad *et al*, 1993), correlating with Corkscrew acting in conjunction with Raf-1 (see **4.2.2** and Pallen *et al*, 1992).

In addition to this direct Ras-mediated signal transduction pathway, treatment of certain cell-types with activators of PKA or PKC, or the interaction of ligand with a G protein-coupled receptor, results in activation of tyrosine phospahatase activity (Pallen *et al*, 1992). Hence, protein tyrosine phosphatase activity has been implicated in various stages of the described signal transduction pathways.

4.3.3 Serine/threonine phosphatases.

An increase in the concentration of intracellular calcium results in loss of CD45 activity, concommitant with loss of a serine phosphate on CD45 (Pallen *et al*, 1992), ie. a feedback loop involving a serine/threonine phosphatase is implicated in this system, and negative regulation of the activity of various cellular kinases, such as PKC, MAP kinase-kinase, MAP kinases and Rsk kinases can be achieved by various serine/threonine phosphatases (Alexander *et al*, 1990; Nakielny *et al*, 1992). Okadaic acid, which specifically inhibits PP-1 and PP-2A (Pallen *et al*, 1992; Alexander *et al*, 1990; Sung *et al*, 1992) can, in certain cell-types, induce the transcription of several early-response genes (Chang *et al*, 1992), correlating with the activation by okadaic acid of transcription factors such as AP-1 and NF-κB (Thevenin *et al*, 1991; Thevenin *et al*, 1990), and suggesting that serine/threonine phosphorylation is required for activation of these factors (see below for further discussion).

The positive involvement of serine/threonine phosphatases in signal transduction is suggested by the fact that the IL-6 and/or IL-1 induction of certain acute-phase genes can be prevented by okadaic acid treatment of hepatoma cells (Ganapathi *et al*, 1992). Further, overexpression of a Ca^{2+}-dependent member of the PP-2B serine/threonine phosphatase sub-family, calcineurin, has been shown to stimulate transcription factor NF-AT-dependent transcription, and the immuno-supressant drugs cyclosporin A (CsA) and FK506 bind to- and inhibit the activity of-calcineurin, and prevent the Ca^{2+}-dependent nuclear translocation of a cytoplasmic component of NF-AT (O'Keefe *et al*, 1992; Clipstone *et al*, 1992; Flanagan *et al*, 1991; Liu *et al*, 1991; Liu *et al*, 1992). Hence, a calcineurin-dependent dephosphorylation event appears to be required for the nuclear translocation of a

cytoplasmic component of NF-AT (O'Keefe *et al*, 1992; Clipstone *et al*, 1992; Flanagan *et al*, 1991; Liu *et al*, 1991; Liu *et al*, 1992). Like PTPases, serine/threonine phosphatases may therefore be involved at several stages, in both positive and negative aspects of signal transduction pathways.

4.4 LPS-STIMULATED SIGNAL TRANSDUCTION.

Lipopolysaccharides (LPS), derived from the cell walls of gram-negative micro-organisms, are among the most potent stimuli leading to the activation of monocytes and macrophages, and induction of MIP1α gene expression in macrophages. These cells display specific cell-surface LPS receptors, and can also absorb LPS by phagocytosis (Chen *et al*, 1989; Wright *et al*, 1990). Furthermore, a variety of other cell types, including erythrocytes and fibroblasts display specific cell-surface LPS receptors (Chen *et al*, 1989; Tannenbaum *et al*, 1990).

There is no information available on LPS-mediated signal transduction in non-monocyte/macrophage cell types. In macrophages, LPS-mediated signal transduction is also poorly understood, however, most investigators appear to agree that LPS stimulation of macrophages leads to rapid activation of PLC-γ, leading to calcium flux and activation of PKC and Cam kinases (Chen *et al*, 1989). Different macrophage cell lines take different times to show maximal response, as shown by measurement of cellular IP_3 levels; for example J774.2 IP_3 levels peak 1 minute after LPS stimulation, while those in P388D1 cells peak after ~20 minutes of LPS stimulation. Some investigators also show that LPS stimulation of macrophages triggers PKC activation by an IP_3-DAG-independent pathway (Chen *et al*, 1989).

Most data suggest that PKA is not involved in the early response of macrophages to LPS stimulation, and in fact it appears that an adenylate cyclase-coupled G_I protein may mediate LPS-induced signal transduction in macrophages, correlating with the observation that LPS-stimulation of macrophages leads to enhanced degradation of prostaglandin-induced intracellular cAMP levels (Okonogi *et al*, 1991). Although there is evidence for the LPS-stimulated activation of other protein kinases in macrophages, the identity of these kinase(s) is at present unknown.

4.5 TRANSCRIPTION FACTORS INVOLVED IN THE MODULATION OF EARLY-RESPONSE GENE ACTIVITY: MODULATION OF THEIR ACTIVITY BY PROTEIN KINASES AND PHOSPHATASES INVOLVED IN SIGNAL TRANSDUCTION.

The modulation of transcription factor activity and hence of transcriptional activity, is clearly an important end-point of the signal transduction pathways that regulate early-response gene expression. Below, I describe some of the transcription factor families involved in the regulation of early-response genes, and the modulation of their activity by some of the protein kinases and phosphatases discussed above.

4.5.1 The Rel family.

The regulatory elements of many early-response genes that are involved in immune responses and/or acute-phase reactions, contain variants of a decameric *cis*-acting motif called κB (Baeuerle, 1991; Grimm *et al*, 1993). A large number of cellular stimuli, including mitogens, primary- (eg. bacteria, oxidative stress) and secondary- (eg. inflammatory cytokines) pathogenic stimuli can signal the modulation of gene expression via the κB motif, via a factor induced to translocate from cytoplasm to nucleus on cellular stimulation, called NF-κB (Baeuerle, 1991; Grimm *et al*, 1993).

Several distinct but closely-related homo- and heterodimeric transcription factor complexes, the components of which are products of genes related to the *c-rel* proto-oncogene, are responsible for this κB-dependent regulation of gene expression (Baeuerle, 1991; Nolan *et al*, 1992; Bours *et al*, 1993). All Rel-family proteins are highly homologous in a region called the RHD (see chapter **2**, section **2.3.10**), required for DNA binding, dimerization and nuclear localization (Grimm *et al*, 1993; Nolan *et al*, 1992; Bours *et al*, 1993).

There are two sub-classes of Rel-family proteins, one containing p50 and p50B (which contain short C-termini), the other containing p65, c-Rel and RelB (Nolan *et al*, 1992; ours *et al*, 1993). An alternatively-spliced form of p65, p65-Δ encodes a protein that, through formation of non-functional heteromeric complexes (greatly reduced DNA binding) may negatively regulate the function of its dimerisation partners. NF-κB appears to mainly consist of p50/p65 heterodimers (Baeuerle, 1991; Nakayama *et al*, 1992), though, as variant κB motifs that show

preference for particular Rel family dimers have been recently been identified, the exact sequence of different κB motifs may be important in determining which Rel-family members regulate a particular gene *in-vivo* (Baeuerle, 1991; Grimm *et al*, 1993; Nolan *et al*, 1992; Bours *et al*, 1993; Ruben *et al*, 1992; Kessler *et al*, 1996; Nakayama *et al*, 1992; Sica *et al*, 1992).

p50 and p50B (also known as p49/p52) (Nolan *et al*, 1992; Bours *et al*, 1993)) are both synthesized as distinct non-DNA binding precursor molecules, p110 and p100 respectively, which contain the N-terminal RHD linked by a glycine-rich region to a C-terminal domain containing multiple ankyrin repeats (Grimm *et al*, 1993; Nolan *et al*, 1992). Proteolytic cleavage of p110 and p100 releases the p50 and p50B proteins (Grimm *et al*, 1993; Nolan *et al*, 1992). p65, RelB and c-Rel possess long transcriptional activation domain-containing C-terminal domains, while p50 and p50B proteins contain only short C-termini(Grimm *et al*, 1993; Nolan *et al*, 1992; Bours *et al*, 1993). As a result, $p50_2$ and $p50B_2$ homodimers appear to be transcriptionally inactive *in-vivo* (Grimm *et al*, 1993; Bours *et al*, 1993), and binding of $p50_2$ at certain κB sites is implicated in negative regulation of positive gene expression mediated by $p65_2$ and NF-κB, presumably by competition for binding at the κB site (Grim *et al*, 1993; Bours *et al*, 1993).

The activity of Rel-family proteins in mammalian cells is controlled by the $I_\kappa B$ family of proteins, which contain potential target sites for protein kinases (see below), and ankyrin repeats which specify their interactions with Rel family proteins (Grimm *et al*, 1993; Nolan *et al*, 1992; Bours *et al*, 1993). Different $I_\kappa B$ proteins interact with overlapping but distinct sets of Rel family proteins, mediating cytoplasmic retention- and prevention/dissociation of DNA binding of Rel family proteins (Grimm *et al*, 1993; Bours *et al*, 1993; Sun *et al*, 1993; Zabel *et al*, 1993). For example, upon cell-stimulation, the $I_\kappa B$ protein MAD-3 is rapidly degraded, releasing cytoplasmic NF-κB, which can then translocate to the nucleus and regulate inducible gene expression (Baeuerle, 1991; Grimm *et al*, 1993; Sun *et al*, 1993; Zabel *et al*, 1993). Interestingly, NF-κB upregulates MAD-3 gene expression, implying that a negative-feedback inhibition occurs (Sun *et al*, 1993). In some cell-types, such as monocytes, MAD-3 is not present and NF-κB is constitutively nuclear (Haskill *et al*,

1991). However, differentiation to macrophages is accompanied by rapid up-regulation of MAD-3 gene expression (Haskill *et al*, 1991).

Many cells, including macrophages, contain abundant nuclear $p50_2$. However, the binding of $p50_2$ to κB sites can be destabilized by Bcl3, which appears to be the only nuclear $I_\kappa B$ (Bours *et al*, 1993). *bcl-3* expression is upregulated upon cell-stimulation, hence displacement of DNA-bound $p50_2$ by Bcl3 and concommitant nuclear translocation and κB site binding of NF-κB could occur (Bours *et al*, 1993). Interestingly, Bcl3 also contains two transactivation domains, and can bind to κB site-bound $p50B_2$, allowing transactivation by the otherwise inactive $p50B_2$ (Bours *et al*, 1993), so that in cells that co-express Bcl3 and p50B, a mechanism for transcactivation via κB sites exists which is independent of p65, c-Rel and RelB (Bours *et al*, 1993).

Apart from the above example, the dissociation of Rel-family members from $I_\kappa B$ proteins may be a prerequisite for nuclear translocation/ DNA binding/ modulation of transcription by Rel-family proteins. As Rel family protein activation occurs in response to a large number of extra-cellular stimuli, it might be expected that multiple signal transduction pathways are able to activate these proteins. Thus, p65, c-Rel, RelB and Dorsal, the Drosophila homologue of this Rel family sub-family, share a PKA phosphorylation consensus site, and phosphorylation of Dorsal by PKA is a prerequisite for nuclear translocation of Dorsal, and enhances its transactivation ability (Norris *et al*, 1992). Both $I_\kappa B\alpha$ and $I_\kappa B\beta$ are inactivated *in-vitro* by treatment with PKA or PKC (Link *et al*, 1992), and *in-vitro* phospatase treatment of $I_\kappa B\beta$ results in loss of it inhibitory activity (Link *et al*, 1992). Further, the p50 precursor (p110) and c-Rel, present together in multi-protein complexes in T-cells, are tyrosine kinase substrates (Lamb *et al*, 1991). Furthermore, addition of the serine/threonine phosphatase inhibitor okadaic acid to intact cells activates NF-κB (Thevenin *et al*, 1990), while tyrosine kinase inhibitors have an inhibitory effect on such induction (Grimm *et al*, 1993).

Activation of NF-κB in response to all inducing agents tested so far is prevented by a variety of anti-oxidants (Baeuerle, 1991; Grimm *et al*, 1993), and cells such as macrophages and neutrophils are known to produce large amounts of intracellular reactive oxygen intermediates (ROIs) in response to inflammatory

stimuli (Baeuerle, 1991; Grimm *et al*, 1993). It is envisaged that ROIs activate various unknown protein kinases, which indirectly mediate the inactivation of $I_{\kappa}B$ molecules by proteolysis or phosphorylation (Grimm *et al*, 1993).

4.5.2 The bZIP class of transcription factors.

The bZIP class of transcription factors is important in the transcriptional regulation of early-response genes. For example, the Fos, Jun and ATF/CREB families of transcription factors bind to both cyclic AMP response element (CRE) and TPA response element (TRE) DNA motifs in the regulatory regions of such genes (Hai *et al*, 1991; Gillespie, 1991; Frame *et al*, 1991), while C/EBP family transcription factors bind to variants of a DNA element found particularly in the regulatory regions of early-response genes involved in immune response and/or acute-phase reactions (Akira *et al*, 1990; Lee *et al*, 1993).

There are at least five Fos-family- (c-Fos, FosB, Fra-1, Fra-2 and Delta-FosB), three Jun-family (c-Jun, JunB and JunD), and thirteen ATF/CREB family-members (Gillespie, 1991; Lamb *et al*, 1991). Fos family members cannot dimerize with other Fos proteins, but dimerise with all of the Jun- and some of the ATF/CREB proteins (Gillespie, 1991; Lamb *et al*, 1991; Hai *et al*, 1991), while Jun proteins can homodimerise, and heterodimerize with Jun-, Fos-, and certain ATF/CREB- proteins (Gillespie, 1991; Lamb *et al*, 1991). ATF/CREB-family proteins can homo- and heterodimerize with *most* ATF/CREB family proteins, and the C/EBP family proteins can homo- and heterodimerize with each other, but not with any of the Fos-, Jun- or ATF/CREB- proteins (Gillespie, 1991; Lamb *et al*, 1991).

Formation of dimers with different partners can alter binding site recognition. For example, dimerization within the ATF/CREB family favours binding to CRE-like DNA elements, Fos- and Jun- protein homo- and heterodimers favour binding to TRE-like elements, and Jun-ATF/CREB heterodimers favour binding to CRE-like elements. In cases where different homo- and heterodimers have similar DNA binding specifity and affinity and are present in the same cell, interaction with proteins bound at neighbouring sites within the context of specific regulatory elements is expected to determine the role of individual homo- and heterodimers in the regulation of the promoter (Lamb *et al*, 1991). Further, different dimers may exist at a particular

regulatory site during transient induction of transcription of early-response genes, as has been shown for different Fos-Jun complexes which exist prior to-, during- and after- cell stimulation (Kovary *et al*, 1992). The reason for this may be due to the fact that different members of these families have different transcriptional activation potential (Kovary *et al*, 1992; Chiu *et al*, 1989; Descombes *et al*, 1991; Hsu *et al*, 1993); for example JunB, by dint of its weak activation potential can act as a negative regulator of the strong transcriptional activator c-Jun in certain promoters (Chiu *et al*, 1989), while the mRNA of the C/EBPβ gene can be translated into three different proteins, two of which contain activation domains and one of which does not (Descombes *et al*, 1991; Hsu *et al*, 1993)

Non-DNA binding bZIP proteins also exist, that have a dominant-negative effect on the normal activities of their dimerization partner. For example, CHOP dimerizes with C/EBP family members and prevents DNA binding (Ron *et al*, 1992), while Δ-FosB is a naturally-occurring truncated form of FosB that inhibits Fos/Jun-mediated transcriptional activation, presumably at the level of dimerization and DNA binding with Jun family members (Nakabeppu *et al*, 1991).

It is apparent that many transcription factors that regulate early response genes are associated with inhibitory molecules prior to the the appropriate cell-stimulation. Thus, c-Fos protein is released from the cytoplasm and translocated to the nucleus upon cellular cAMP treatment; while cell-specific negative regulators of c-Jun activity have been identified; a protein IP-1 has been identified that interacts with AP-1 complexes and prevents AP-1 DNA binding (Auwerx *et al*, 1992). IP-1 is inactivated *in-vitro* by PKA, and *in-vivo* by PKC- PKA- and Ca^{2+}-dependent kinase signal transduction pathways (Auwerx *et al*, 1992). Cytoplasmic negative regulators of C/EBP family members are implied, in that (at least in some cell-types) C/EBPβ becomes phosphorylated, translocates from cytoplasm to nucleus and activates transcription in a cAMP-dependent manner (Metz *et al*, 1991a).

Protein kinases and phosphatases also directly modify bZIP transcription factors. For example, both c-Jun and C/EBPβ are both serine/threonine phosphorylated in a Ras-dependent manner (Nakajima *et al*, 1993; Smeal *et al*, 1993). c-Jun serine phosphorylation within its activation domain is greatly enhanced- and at three negative-regulatory (when phosphorylated) sites is greatly decreased-, upon

cell-stimulation with different activated oncoproteins corresponding to transmembrane RTKs, Src, Ras or Raf-1, ie. all proteins at different points in the Ras-mediated signal transduction pathway discussed earlier (Smeal *et al*, 1993). MAP kinases, Casein kinase II and PKC are also implicated in the pathway mediating the dephosphorylation-dependent activation of c-Jun (Ahmad *et al*, 1993; Chou *et al*, 1992; Lin *et al*, 1992; Boyle *et al*, 1991).

Transactivation by C/EBPβ is also stimulated by activated Ras (Nakajima *et al*, 1993), and this stimulation is achieved by threonine phosphorylation of C/EBPβ at a site next to the DNA binding domain (Nakajima *et al*, 1993), which is phosphorylated *in-vitro* by purified MAP kinases (Wegner *et al*, 1992). C/EBPβ can also be phosphorylated within its leucine zipper in a calcium-dependent manner, possibly by Cam Kinase II, leading to an increase in the ability of C/EBPβ to stimulate transcription (Wegner *et al*, 1992). It is speculated that this may be achieved by inducing a change in C/EBP family dimerization partner of C/EBPβ (Wegner *et al*, 1992), though it could perhaps modulate C/EBPβ interaction(s) with non-C/EBP family members (Lamb *et al*, 1991; LeClair *et al*, 1992).

From this discussion, and similar information on other proteins that dimerize with c-Jun and C/EBPβ, it is evident that, depending on cell-type, perhaps all of the signal transduction pathways discussed earlier are able to mediate transcriptional induction via Fos/Jun and C/EBP family members.

4.5.3 Post-translational modification of transcription factors by direct tyrosine phosphorylation.

Upon cellular IFN α/β treatment, a non-receptor tyrosine kinase, probably Tyk-2 (*not* related to Src), tyrosine phosphorylates the three cytoplasmic components (collectively termed ISGF3α) of the ISGF3 transcription factor, which translocate to the nucleus and become associated with a nuclear protein called ISGF3γ (Fu, 1992; Schindler *et al*, 1992; Velazquez *et al*, 1992). ISGF3γ alone binds with low affinity to a promoter element called the interferon stimulated response element (ISRE), but on association with ISGF3α, an active ISGF3 complex is formed which has a 20-fold higher affinity for the ISRE (Fu, 1992; Schindler *et al*, 1992).

Pretreatment of several cell types with IFNγ has been shown to increase the available levels of ISGF3γ, thus allowing subsequently greater induction of ISRE-

containing genes in response to subsequent cellular IFN α/β treatment (Fu, 1992; Schindler *et al*, 1992; Velazquez *et al*, 1992). In certain cell-types, IFNγ appears to activate transcription by stimulating the tyrosine phosphorylation and subsequent nuclear translocation of only the p91 subunit of ISGF3α, presumably by activating a kinase different from Tyk-2 (Schindler *et al*, 1992). Hence, direct tyrosine phosphorylation- and concommittant activation of- cytoplasmic transcription factors may be fairly common.

4.6 POST-TRANSCRIPTIONAL AND POST-TRANSLATIONAL MECHANISMS REGULATING EARLY-RESPONSE GENE PROTEIN PRODUCTION.

In addition to the role of transcription in the accumulation of early-response gene mRNA in response to cell stimulation, post-transcriptional- and post-translational mechanisms also contribute to the regulation of early-response gene expression.

4.6.1 Coupling of mRNA stability and translational control.

Prior to cell-stimulation, early-response gene mRNAs are very unstable, with half-lives typically of less than thirty minutes (Vakalopolou *et al*, 1991). Upon the appropriate cell-stimulation, the mRNAs of these genes become transiently stabilized (Vakolopolou *et al*, 1991; Brewer 1991). The 3' untranslated regions (UTRs) of early response genes' mRNAs, including those encoding many cytokines and proto-oncogenes (including MIP1α), contain conserved AU-rich motifs, and it was originally shown that a 51 base AU-rich domain from the GM-CSF mRNA 3'UTR could reduce the half-life of a previously stable β-globin mRNA to thirty minutes (Shaw *et al*, 1986; Cleveland *et al*, 1989; Peltz *et al*, 1992). Such mRNA instability conferred by 3'UTR sequences may be cell-specific and, further, particular cell-types can differentially regulate the stability of different mRNAs via their 3'UTR sequences (Bohjanen *et al*, 1991). Thus, in experiments in which the 3'UTR sequences of different genes were linked to the *neo* reporter gene, GM-CSF 3'UTR hybrid transcripts and endogenous mRNA are stable in monocytes but unstable in fibroblasts, while *c-myc* and *c-fos* hybrids and endogenous mRNAs are unstable in both cell-types (Bohjanen *et al*, 1991).

A cytoplasmic factor called AU-B, which is induced in an RNA- and protein synthesis- dependent manner upon TCR stimulation of T cells, binds cytokine- but not *c-myc*- 3'UTR AU-rich sequences (Bohjanen *et al*, 1991). Notably, stabilization of the cytokine mRNAs correlated inversely in this study with AU-B binding activity (Bohjanen *et al*, 1991). Various other proteins which bind to 3'UTR AU-rich sequences and which may be involved in their function, have also been described (Peltz *et al*, 1992).

3'UTR AU-rich instability elements (AREs) appear to confer rapid removal of the polyA tail, which requires an ARE but not specifically the conserved AUUUA motif, then the subsequent decay of the body of the transcript, which *does* require the conserved AUUUA motif of the ARE (Chen *et al*, 1992). Different proteins isolated that bind AREs appear to specifically bind to *either* the conserved AUUUA sequence *or* the other AU-rich sequences (Peltz *et al*, 1992).

AREs also appear to mediate the efficiency of translation of such mRNAs, as they confer a translational block, which can be relieved upon the appropriate cell-stimulation (Han *et al*, 1990; Kruys *et al*, 1989). It appears that this translational block may be due to deadenylation of the transcripts, and presumably in certain cell-types, ARE binding proteins are present that direct de-adenylation, but those required for subsequent degradation of the transcript are not present (Kruys *et al*, 1989; Huarte *et al*, 1992).

The protein synthesis inhibitor cycloheximide is noted for its ability to up-regulate early-response gene mRNA levels, and prolong the normally transient period of early-response gene mRNA accumulation (Laird-Offringa *et al*, 1990). Cycloheximide has been shown to prevent the polyA tail shortening step of the early-response gene ARE-directed mRNA degradation pathway (Laird-Offringa *et al*, 1990). Hence, the requirement of translation for ARE-directed mRNA instability (Cleveland *et al*, 1989; Peltz *et al*, 1992) could be attributed to the fact that the ARE binding protein AU-B is synthesized *de-novo* upon cell-stimulation, and is therefore not synthesized in cycloheximide-treated cells (Bohjanen *et al*, 1991).

In at least some cell-types, cell-stimulation leads to transient early-response gene mRNA accumulation, but no concommittant translation of those mRNAs (Schindler *et al*, 1990). For example, in monocytes, IL-1β mRNA can be transiently

induced in the absence of detectable IL-1β protein synthesis (Schindler *et al*, 1990). However, when LPS is used as the stimulus, IL-1β mRNA *and* protein synthesis are both induced (Schindler *et al*, 1990). This suggests that certain cell stimuli cannot inactivate the polyA-removal step of the 3'UTR ARE-directed mRNA degradatory pathway, but can inactivate the subsequent ARE-directed degradation of the body of the transcript. In the above example, LPS induction correlates with the inactivation of both the polyA-removal *and* degradatory steps of the early-response gene ARE-directed mRNA degradation pathway.

Finally, it must be stressed that it may be generally applicable that early-response gene mRNA degradation occurs via at least two separate pathways, only one of which is directed by 3'UTR AREs (Brewer, 1991; Chen *et al*, 1992; Han *et al*, 1991). The mRNA degradatory pathways specified by these other elements appear to be distinct from those directed by 3'UTR AREs, though may direct degradation by a similar mechanism to the 3'UTR AREs in at least some cases (Brewer, 1991; Chen *et al*, 1992; Han *et al*, 1991).

4.7 TRANSCRIPTIONAL ELONGATION AND EUKARYOTIC GENE REGULATION.

This manuscript is primarily concerned with the regulation of transcriptional *initiation* of eukaryotic genes. However, it is becoming apparent that regulation of transcriptional elongation may also be an important mechanism in the control of early-response gene transcription (Spencer *et al*, 1990). For example, in the case of the *c-fos* gene in primary murine macrophages, a block to transcriptional elongation in the first intron is relieved in a calcium-dependent manner (Collart *et al*, 1991), while in the *c-myc* gene, a block to transcriptional elongation near the end of exon 1 can be relieved by phorbol esters (PKC activators) (Lindsten *et al*, 1988). Hence, signal transduction pathways which alter transcriptional initiation can also modulate transcriptional elongation.

CHAPTER 5: GENE EXPRESSION WITHIN CHROMATIN.

5.1 OVERVIEW.

In the preceding pages, transcriptional regulation of higher-eukaryotic genes has been described, with only passing reference to the fact that within any cell, the majority of the DNA is tightly condensed into chromatin. In the following section, chromatin is described, and mechanisms for the regulation of gene expression within chromatin are discussed.

5.2 WHAT IS CHROMATIN ?

In eukaryotic cells, a large amount of DNA is packaged into the nucleus of a comparatively small cell. Thus, the diploid human genome contains $6x10^9$ bp of DNA which, if stretched out would span ~1 metre. Packaging of chromosomes into the nucleus therefore requires a high degree of compaction of the DNA, and involves several hierarchical levels, in which DNA is associated in a nucleoprotein complex termed chromatin.

The fundamental repeating unit of chromatin is the nucleosome (McGhee *et al*, 1980; Richmond *et al*, 1984), which contains a histone octamer, consisting of two each of histone proteins H2A, H2B, H3 and H4, arranged as dimers in the order (H2B/H2A)-(H4/H3)-(H3/H4)-(H2A/H2B) (McGhee *et al*, 1980; Richmond *et al*, 1984; Kornberg *et al*, 1992). 146bp of negatively supercoiled DNA is wound 1.75 times around the octamer, to form the nucleosomal core particle (McGhee *et al*, 1980; Richmond *et al*, 1984; Kornberg *et al*, 1992; Morse, 1992). Nucleosomes are present along the entire length of nuclear DNA, separated by up to ~50 bp of "linker" DNA , giving the chromatin a "beads-on-a-string" appearance (McGhee *et al*, 1980); this is the 10 nm chromatin fibre.

Histone protein H1 (H5 in birds, which will not be discussed), binds to each nucleosome and associated linker DNA near where the DNA helix enters and leaves the histone octamer, locking two full turns, or 166 bp of DNA, around each nucleosomal core in one negative supercoil (Grunstein, 1990). Inter-nucleosomal interactions, mediated by histone H1, allows the chromatin to be condensed into a 30nm fibre, though the exact structure of the 30nm fibre is not known (Allan *et al*, 1981; Felsenfeld *et al*, 1986).

In order for the DNA to fit into the cell nucleus, a further 100-200-fold compaction is required, giving *higher-order* chromatin. Evidence suggests that the 30nm fibre is attatched in a series of discrete and topologically independent loops (radial loops) to scaffold proteins within the chromosome, whereby the base of each loop is attatched to the scaffold protein (Ratner *et al*, 1985).

5.3 ACTIVE AND INACTIVE CHROMATIN.

5.3.1 Overview.

The majority of genes in any cell-type are inactive. Given that chromosomal DNA is incorporated into a highly condensed higher-order structure, which is liable to make the DNA essentially inaccessible to gene-regulatory proteins, a major challenge has been to investigate how *any* gene within such a structure is active. The activation status of a gene (ie. whether it is transcribing/ ready to transcribe mRNA) appears to be associated with four inter-linked phenomena, which are described below.

5.3.2 DNA Replication.

S-phase, the phase of the cell-cycle during which the DNA in a higher eukaryotic cell is replicated, spans approximately 8-12 hours. The replication of chromosomal DNA during this period can be studied using pulse-labelling with the thymidine analogue BrdU, which differentially fluoresces in cytological analysis (Hand, 1978). These studies show that a series of replication bands appear in a reproducible programmed manner along the whole length of the chromosome; an average human chromosome, for example, contains about fifty replication bands, each of which contains several megabases of DNA and approximately 10-20 origins of DNA replication that are co-ordinately activated (Hand, 1978; Fangman *et al*, 1992).

Examination of the replication times of individual genes, using a combination of the BrdU technique, sedimentation analysis and Southern blotting (Braunstein *et al*, 1982), reveals that almost all housekeeping genes are replicated within the first half of S-phase, while most tissue-specific genes replicate early in S-phase in cells in which they are expressed, but late in S-phase in non-expressing cell-types (Hatton *et al*, 1988; Forrester *et al*, 1991). Further, many tissue-specific genes change from late- to early-S-phase replication when they become competent for expression (Hatton *et al*, 1988).

In the case of multi-gene families, for example the β-globin locus, where all the genes are at the same locus, all genes are co-replicated, regardless of transcriptional activity (Hatton *et al*, 1988; Forrester *et al*, 1991). Notably, the erythroid-specific genes of the α-globin locus are replicated early in S-phase in both expressing and non-expressing cells (Forrester *et al*, 1991; Vyas *et al*, 1992). However, the α-globin genes, unlike most tissue-specific genes, are situated in a chromosomal region densely packed with housekeeping (ie. early replicating) genes (Vyas *et al*, 1992; Forrester *et al*, 1991).

It appears that chromosomal context is important in determining the replication time of a gene. This is well demonstrated by the mammalian X-chromosome: in females, one copy of the X-chromosome is condensed in heterochromatin, and is transcriptionally inactive, while the other is not. The inactive chromosome is replicated late in S-phase, while homologous genes on the active X-chromosome are replicated early in S-phase (Fangman *et al*, 1992).

5.3.3 DNaseI sensitivity and hypersensitivity:

5.3.3.1 Overview:

Enzymes such as DNaseI, which nicks one strand of double-stranded DNA in a pseudo-random manner, have been used to investigate the accessibility of chromatin (Gross *et al*, 1988). In general, chromatin is relatively resistant to DNaseI cleavage; however, large regions of chromatin, for example ~200 kbp in the case of the β-globin locus (Evans *et al*, 1990) and >20 kbp in the case of the chicken lysozyme gene (Elgin, 1990), are approximately an order of magnitude more sensitive to DNaseI cleavage in expressing cell-types than is bulk chromatin (Gross *et al*, 1988), corresponding to regions that contain actively- or potentially-actively (poised) transcribed genes (Gross *et al*, 1988; Evans *et al*, 1990; Elgin, 1990; Elgin, 1988). Certain other discrete (of the order of 50-400 bp) sites within DNaseI-sensitive chromatin are *hypersensitive* (typically two orders of magnitude more sensitive than bulk chromatin) to DNaseI cleavage (Gross *et al*, 1988; Evans *et al*, 1990; Elgin, 1990; Elgin, 1988).

In all observed cases, chromatin DNaseI hypersensitive sites correspond to functional sequences; these include promoters, enhancers, silencers, origins of DNA replication, and at least some potential chromatin boundary elements (see below),

such as Locus Control Regions (LCRs) and Special Chromatin Sequence (scs) elements (Gross *et al*, 1988; Evans *et al*, 1990; Elgin, 1990; Elgin, 1988; Kellum *et al*, 1992; Stief *et al*, 1989). Like the activity of the functional sequences to which they map, such hypersensitive sites can be constitutive, inducible, developmentally-regulated or tissue-specific (Gross *et al*, 1988).

Analysis of the fine structure of DNase1 hypersensitive sites (DHSs) reveals "hot" and "cold" spots, regions of extreme DNase1 sensitivity (hypersensitivity) flanking sites of comparative DNase1 insensitivity (Gross *et al*, 1988). The hypersensitivity correlates with lack of a nucleosomal core particle(s), while the intra-hypersensitive site "cold" spots are due to binding of *trans*-acting non-histone proteins, as demonstrated by the *in-vivo* genomic footprinting technique (Gross *et al*, 1988).

5.3.3.2 Constitutive hypersensitivity.

Housekeeping genes, which are constitutively active, contain constitutive hypersensitive sites over their promoter regions (Gross *et al*, 1988). Early-response genes, as exemplified by *c-fos* or *c-myc*, also have constitutive promoter hypersensitive sites, which do not detectably change upon induction of transcription (Herrera *et al*, 1989; Gibson *et al*, 1992; Renz *et al*, 1985). This suggest that the chromatin around such genes, when uninduced, is "poised", allowing for rapid induction of transcription, in the absence of DNA replication, in response to the appropriate extra-cellular signal (Herrera *et al*, 1989).

5.3.3.3 Inducible hypersensitivity:

Apart from induction of transcription by steroid hormones (Workman *et al*, 1988; Archer *et al*, 1991; Archer *et al*, 1992), there appear to be no examples of the induction of chromatin DNase1 hypersensitivity in the absence of DNA replication (except when the histones bound to a region of DNA are potentially acetylated: see chapter 5, section 5.3.5.1, plus Verdin *et al*, 1993), presumably due to the inability of most transcription factors to recognise their binding site on DNA when the DNA is complexed with unmodified nucleosomes (Workman *et al*, 1988).

The special ability of steroid hormone receptors (SHRs) to rapidly induce DNase1 hypersensitivity upon hormone stimulation is exemplified by the studies of glucocorticoid receptor (GR) induction of the MMTV promoter. The GR is a ligand-

dependent DNA-binding transcription factor that has a recognition site in the MMTV promoter (Archer *et al*, 1991). This promoter is complexed with six positioned nucleosomes (nucleosomes do not take up entirely random positions on DNA; positioning is dependent on DNA sequence, presence of other nucleosomes,etc. (Drew *et al*, 1987)) when stably introduced into mammalian cells, so that transcription factor binding sites on the promoter DNA are complexed with the nucleosomes (Archer *et al*, 1991).

One nucleosome (nucB) is positioned over binding sites for a group of transcription factors involved in promoter activation, including GR, NF-1, and Oct-family proteins (Archer *et al*, 1991; Archer *et al*, 1992; Bruggermeier *et al*, 1991). As GR can recognise its nucleosomally-bound binding site on the MMTV promoter with high sppecificity (Archer *et al*, 1991; Perlmann *et al*, 1992), binding of GR to this nucleosomal site displaces nucB, and hence induces DNase1 hypersensitivity, exposing the overlapping NF-1 recognition site (Archer *et al*, 1991). Interestingly, the GR and NF-1 factors appear to compete for binding to DNA, and transient transfections in which NF-1 is shown to be critical for inducible activity of the MMTV promoter (Bruggermeier *et al*, 1990) take no account of the fact that NF-1 is constitutively bound to the MMTV promoter in transient transfections but is unable to access the nucleosomally-bound DNA prior to hormone treatment in stable transfectants (Bruggermeier *et al*, 1990). In contrast, GR displacement of nucB appears to allow co-operative binding of Oct-1 transcription factor to the MMTV promoter, allowing hormone-inducibility (Bruggermeier *et al*, 1991).

5.3.3.4 Tissue-specific hypersensitivity.

The tissue-specificity of promoter/enhancer DNase1 hypersensitivity corresponds to the tissue-specificity of gene expression (Gross *et al*, 1988; Evans *et al*, 1990; Elgin, 1988). This is well-illustrated by the chicken lysozyme gene, which is primarily expressed in the oviduct and in macrophages (Elgin, 1988). In these cell-types, >20 kbp of chromatin surrounding the gene is preferentially DNase1-sensitive, and contains multiple DNase1 hypersensitive sites which map to lysozyme gene regulatory sequences (Elgin, 1988). Two of these sites correspond with positions of known functional promoter and enhancer sequences, and in all non-expressing tissues, these two hypersensitive sites are not present (Elgin, 1988). Several hypersensitive

sites associated with the lysozyme gene *are* present in non-expressing tissues, at least one of which functionally correspond to a "silencer" element in certain tissues (Elgin, 1988).

5.3.3.5 Developmentally-associated DNase1 hypersensitivity.

In humans, the genes of the β-globin multi-gene locus are expressed exclusively in cells of the erythroid lineage in which, in contrast to non-expressing cells, the whole locus of >100 kbp is preferentially DNase1 sensitive (Evans *et al*, 1990). As shown in figure **5.1**, the ε-globin gene is the proximal- and the β-globin- the distal gene of the locus, and five erythroid-specific DNase1 hypersensitive sites (HSI to HSV) are present 5' of the ε-globin gene and one erythroid-specific DNase1 hypersensitive site (HSVI) is present 3' of the β-globin gene in embryonic, foetal and adult cells, ie. these hypersensitive sites are developmentally stable (Forrester *et al*, 1990). The general DNase1 sensitivity of the locus and the developmentally-stable hypersensitive sites are already established in primitive erythroid cells *prior* to detectable globin gene transcription (Forrester *et al*, 1990). HSs -II and -III have been demonstrated to have enhancer function with respect to genes of the β-globin locus (Evans *et al*, 1990; Kellum *et al*, 1992; Tuan *et al*, 1989).

Of the β globin-like genes, the ε globin gene is expressed only in the embryo, the γ globin genes in the foetus, and δ- and β globin genes in the adult (Evans *et al*, 1990). Investigation of the chromosomal conformation of the γ-, δ- and β globin genes reveals that foetal erythroid cells display DNase1 hypersensitive sites closely-associated with all four of these genes, while adult erythroid cells display only the δ- and β globin gene-associated hypersensitive sites (Groudine *et al*, 1983), ie. promoter DNase1 hypersensitivity of these genes is developmentally regulated.

The above discussion illustrates that DNase1 hypersensitive sites are associated with functional gene regulatory sequences such as promoters, enhancers and silencers, and reflect the constitutive, induced, tissue-specific and developmentally-regulated gene expression conveyed by the regulatory sequences over which they are situated.

5.3.3.6 Transcriptional activity requires DNase1 hypersensitivity of gene regulatory sequences.

Unlike DNA-methylation/ demethylation (see section **5.3.5**), which appears to be regulated as a *consequence* of modulation of activity of a gene, activation of transcription appears to be *preceded* by appearance of hypersensitive site(s) associated with a gene. For example, appearance of the developmentally-stable hypersensitive sites associated with the β globin locus, the β globin gene promoter DNase1 hypersensitive site, and the hypersensitive sites associated with the chicken vitellogenin gene, precedes transcripitional activity of the associated genes (Elgin, 1988; Forrester *et al*, 1987; Groudine *et al*, 1983; Burch *et al*, 1983). These observations correlate with the need for promoter/ enhancer-bound transcription factors in order for transcription to proceed, as discussed in the chapter **3**.

5.3.3.7 Locus Control Region.

In functional studies of gene regulatory sequences, using stable transfections or transgenic mice, it is generally the case that highly-variable expression of the exogenous gene is obtained, which does not correlate with the integrated copy number of that gene (Grosveld *et al*, 1987). For example, when the human β-globin gene, including promoter plus intragenic enhancer and 3' enhancer elements, is used to generate transgenic mice, the level of expression obtained is at best an order of magnitude lower than that of the endogenous gene, and 70% of the transgenic animals do not even express detectable levels of the transgene (Grosveld *et al*, 1987). The highly variable levels of expression obtained presumably reflects the chromatin environment into which the construct is integrated, in addition to the absence of critical regulatory sequences (Forrester *et al*, 1990; Grosveld *et al*, 1987; Ryan *et al*, 1989b). In contrast, transgenic mice containing the region of the human β globin locus encompassing the 5' developmentally stable erythroid-specific DNase1 hypersensitive sites, express high levels of a linked human β globin- or heterologous reporter gene mRNA in a position-independent, erythroid-specific manner (Forrester *et al*, 1990; Grosveld *et al*, 1987; Ryan *et al*, 1989b; van Assendelft *et al*, 1989). This region has therefore been named the Locus Control Region (LCR) of the β globin locus (Forrester *et al*, 1990; Grosveld *et al*, 1987).

In the above experiments, constructs containing the region of DNA encompassing 3' HSVI (see figure **5.1**) in addition to the LCR, were expressed no better than constructs containing the LCR without this region (Grosveld *et al*, 1987).

Similarly, some investigators provide evidence that 5'HSs II can act individually as an LCR (Ryan *et al*, 1989b), though others have shown that this 5' HS in *single-copy* form cannot function as an LCR (Ellis *et al*, 1993), and all of the LCR HSs are needed in combination for full LCR activity in transgenic mice (Ryan *et al*, 1989b; Collis *et al*, 1990). Indeed, a β-thalassemia patient whose DNA contains a deletion encompassing four of the five HSs of the LCR (the "Hispanic" deletion: see figure **5.1**), provides evidence that 5'HSI plus 3' HSVI are insufficient for LCR activity (Forrester *et al*, 1990; Grosveld *et al*, 1987).

Of the regions in the LCR, HSs II and III behave as strong enhancers individually in transfection studies, while the other sites show no enhancer activity either individually or in combination (Ryan *et al*, 1989b; Collis *et al*, 1990). The sequences corresponding to HSs-II, -III and -IV bind similar tissue-specific and ubiquitous transcription factors to those that bind the promoters and enhancers of genes of the β globin locus (Strauss *et al*, 1992; Liu *et al*, 1992; Lowrey *et al*, 1992;Talbot *et al*, 1991; Pruzina *et al*, 1991; Lavelle *et al*, 1991; Ellis *et al*, 1993).

The fact that single-copy HS-II constructs cannot function as LCRs, but multi-copy concatomers can (Ellis *et al*, 1993), that all four 5' HSs are needed for full LCR activity, plus the fact that LCR sites share binding sites for transcription factors with each other and with β globin-like gene promoter and 3' enhancer elements has led to the suggestion that the LCR HSs interact with each other through DNA-bound protein-protein interactions, before interacting as a single unit with the appropriate β globin-like gene via promoter/enhancer-bound transcription factors (Ellis *et al*, 1993).

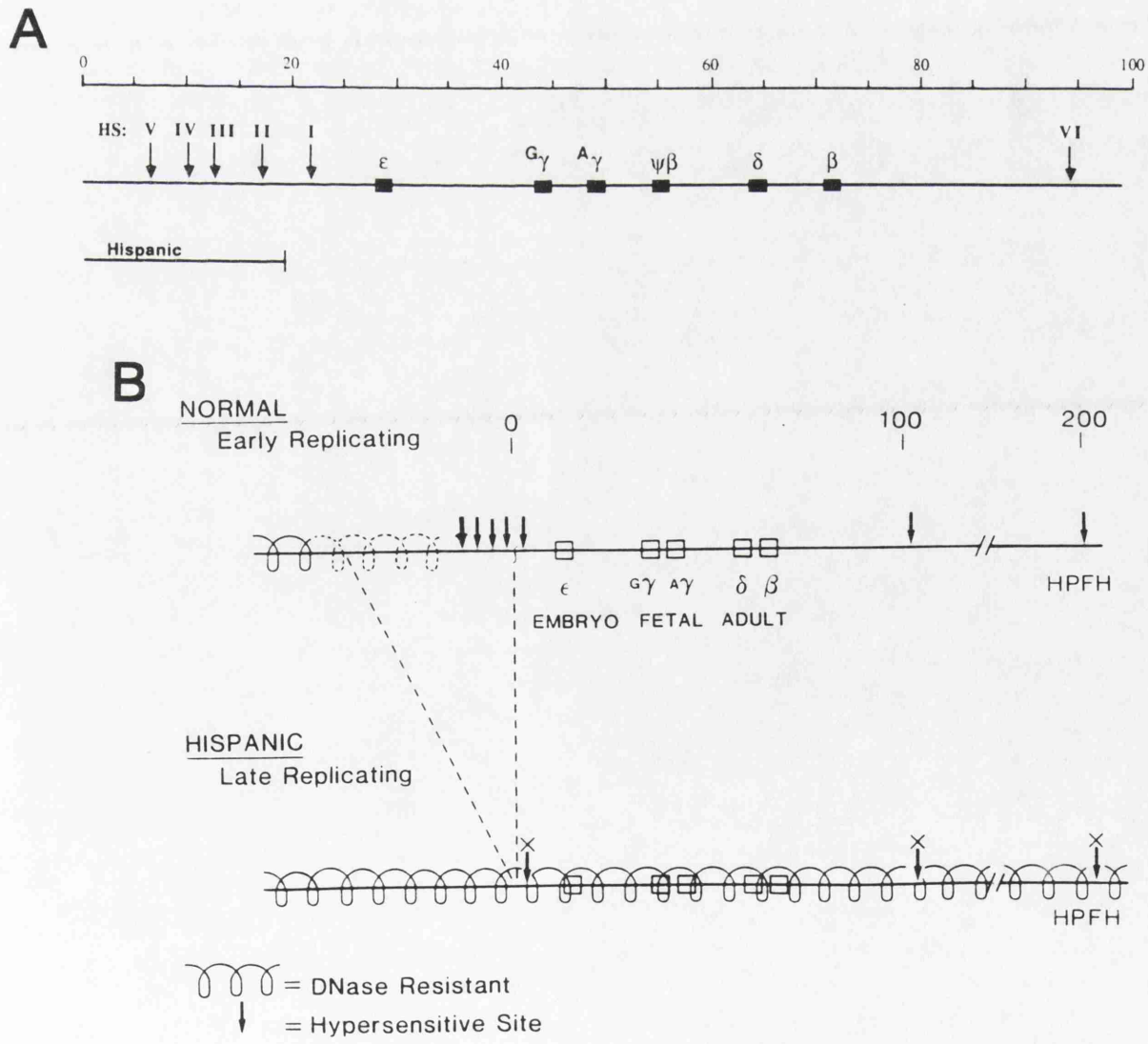
5.3.4 Boundary Elements.

5.3.4.1 Overview.

The 30nm chromatin fibre appears to be organised in a series of discrete and topologically-independent loops (Rattner *et al*, 1985). In addition to allowing the compaction of chromatin, various investigators have proposed that these higher-order domains, generally ~5-100 kbp in size, correspond to units of gene regulation, as manifested by the polytene chromosome "puffs" seen in Drosophila (Kellum *et al*, 1992; Ericsson *et al*, 1990). Such topologically-independent units of gene regulation might act to constrain adventitious activation of genes in one domain by regulatory elements present in a different domain; ie. such domain organisation might prevent

Figure 5.1: The human β-globin gene locus.

A: Schematic diagram of the human β-globin gene locus. A 100 kbp region of human chromosome 16 containing the β-like globin genes is illustrated. Erythroid-specific DNase1 hypersensitive (HS) sites I-V located 6-22 kbp 5' of ε and VI located 19 kbp 3' of β are marked by arrows. The line beneath the locus represents the deletion involved in Hispanic γδβ-thalassemia, as described in the text. **This figure is derived from Ryan *et al*, (1990). B**: Erythroid-specific pattern of DNase1 sensitivity, timing of DNA replication, and location of developmentally-stable hypersensitive sites (corresponding to sites I to VI in **A**)are shown for the β-like globin gene locus and flanking regions in normal and Hispanic chromosomes. Relative positions of the β-like globin genes, developmentally-stable hypersensitive sites, the HPFH region (see text), and the 5' boundary of DNase1-sensitive chromatin are shown. Undetected hypersensitive sites in the Hispanic thalassemia are indicated by arrows with X above them, DNase1-resistant chromatin is represented by coiled lines, and DNase1-sensitve chromatin is represented by straight lines. The dashed coil in the normal chromosome shows the boundary region between DNase1-sensitive and DNase1-resistant chromatin in the 5' flanking region. **This figure is derived from Forrester *et al* (1990).**



the looping-out-mediated interaction, over large distances, of certain enhancers and the wrong promoter element(s) (Kellum *et al*, 1992). It is believed that chromatin is organised into these domains by certain nucleoprotein structures assembled at specific sites along the chromosome, namely boundary elements.

5.3.4.2 The β globin LCR.

It is generally believed that protein-protein interactions mediate the looping-out between LCR components, between the LCR (HSs I-V) and the 3' HSVI (figure **5.1**), and between LCR and promoters/ 3'enhancers, thus accounting for the activity of the LCR as a super-strong enhancer (Ellis *et al*, 1993). However, further evidence suggests that the LCR may act as a boundary between chromatin domains, and that this function may be achieved in a manner distinct from the described looping-out mechanism.

In both murine and human β globin loci, the tissue-specific and developmentally-stable 5' and 3' HSs and the concommittant DNase1-sensitivity of the locus (figure **5.1**) is established *prior* to overt globin gene transcription (Forrester *et al*, 1990; Jimenez *et al*, 1992). This DNase1 sensitivity is entirely dependent on the LCR, as a naturally-occurring deletion (the "Hispanic" deletion: figure **5.1**) of the LCR results in the entire β globin locus plus *at least* as far as 130 kbp 3' of the β globin gene (at *least* to the HPFH gene locus, which is normally DNase1 sensitive- and contains a DNase1 hypersensitive site- in an erythroid background: figure **5.1**), ie. >180 kbp 3' of the LCR, becoming DNase1 resistant in an erythroid background (Forrester *et al*, 1990) (figure **5.1**).

As would be expected of cell-specific genes, the β globin locus is normally replicated early in S-phase in erythroid cells, but late in S-phase in non-erythroid cells (Forrester *et al*, 1990). Sequences up to 30 kbp 5' of the LCR are also replicated early in S-phase in erythroid cells, though they are DNase1 resistant (figure **5.1**). However, when the LCR is deleted, the latter region, plus the whole of the β globin locus, plus >100 kbp downstream of the β globin gene that was DNase1 sensitive and early-replicating is now replicated *late* in S-phase in an erythroid cell background (Forrester *et al*, 1990) (figure **5.1**). Hence, the LCR affects chromatin structure over >180 kbp unidirectionally in a 3' direction, and affects DNA replication bi-directionally over

>200 kbp (suggesting the LCR or sequences close thereof contain origin(s) of DNA replication) (figure **5.1**).

As the region of chromatin immediately 5' of the LCR is DNase1 resistant in erythroid cells, it appears that the LCR blocks the spread of DNase1 resistant chromatin further dowstream. It is difficult to envisage how a looping-out enhancer-like mode of action of the LCR would account for the DNase1-sensitivity of the whole > 200 kbps region 3' of the LCR, as when transcription of subsets of genes of the locus is occurring, for example the ε-globin gene, models suggest looping out between the relevant promoter and the LCR; meanwhile the rest of the locus, >100 kbp, is *not* looped-out but remains DNase1 sensitive.

Even a model wherein the DNase1 sesitivity of the locus is maintained by a looping-out mechanism, involving the LCR, 3'HSVI and the various promoters and enhancers of the β globin locus, cannot explain why >100 kbp 3' of the 3'HSVI is DNase1 sensitive in an LCR-dependent manner in an erythroid cell background (Forrester *et al*, 1990) (figure **5.1**). Hence the LCR appears to act as a *boundary* between chromatin domains (presumably the directionality comes from interaction of the LCR with elements within the β globin locus).

5.3.4.3 The Su(Hw) protein and its interaction with potential boundary elements.

In Drosophila, certain tissue-specific mutations are caused by insertional mutagenesis by a retrotransposon called "gypsy", the phenotypes of which are reversed by mutation in the gene encoding the su(Hw) protein (Roseman *et al*, 199 3). Multimers of the ubiquitous su(Hw) protein bind to a specific region of the "gypsy" element that contains twelve copies of an octamer-like motif, all of which are required for "gypsy" mutagenesis (Roseman *et al*, 1993). In addition to DNA binding, the su(Hw) protein contains zinc-finger motifs, a leucine zipper and two acidic regions, all of which are characteristic of eukaryotic transcription factors (Roseman *et al*, 1993). Insertion of the su(Hw) binding region of the "gypsy" element *between* the enhancer and promoter of a test gene, even if the distance between enhancer and promoter is many kbps, results in inactivation of the effects of that enhancer, while insertion outside of this region does not result in blocking of enhancer function (Roseman *et al*, 1993; Geyer *et al*, 1992). Further if a test gene is flanked by the su(Hw) binding element, the gene is expressed in a position-independent manner in

euchromatin, suggesting that the su(Hw) binding element protects the gene from both positive and negative effects (enhancers/ silencers) (Roseman *et al*, 1993).

Several mechanisms for the described enhancer-blocking effect have been proposed (Roseman *et al*, 1993; Geyer *et al*, 1992). Assuming the looping-out mechanism of action of enhancers, it is suggested that proteins bound at the su(Hw) binding region could interact with promoter- or enhancer-bound factors, or with adaptor proteins that normally mediate communication between enhancer and promoter (Roseman *et al*, 1993; Geyer *et al*, 1992). However, as the enhancer-blocking only occurs when the su(Hw) binding region is *between* enhancer and promoter, this appears to discount the adaptor-, promoter- and enhancer-interference hypotheses (Roseman *et al*, 1993).

Another proposal that fits the described data, is that that the su(Hw) protein is involved in the establishment of a *boundary* of higher-order chromatin (Roseman *et al*, 1993; Geyer *et al*, 1992). Further evidence for this has come from the study of Position Effect Variegation (PEV) in Drosophila wherein, in the vicinity of heterochromatic regions, differential spreading of heterochromatin condensation results in variable inactivation in different cells of a gene inserted close to the region of heterochromatin (Henikoff *et al*, 1992). The presence of a su(Hw) binding site reduces the PEV observed with test genes inserted next to heterochromatic regions, and mutation in the su(Hw) gene results in enhanced PEV (Roseman *et al*, 1993). This suggests that the su(Hw) protein can be involved in the formation of boundary elements between chromatin forms (Roseman *et al*, 1993; Geyer *et al*, 1992).

Characteristics of the su(Hw) binding region of "gypsy" are similar to those of other elements suggested to be boundary elements, such as the *scs* and *scs'* elements of the Drosophila 87A7 heat shock locus (Kellum *et al*, 1992) and the 5' A element which normally resides at the 5' border of the DNase1 sensitive domain surrounding the chicken lysozyme gene (Elgin, 1990; Stief *et al*, 1989), though the *scs* and *scs'* elements do not appear to contain su(Hw) binding sites (Kellum *et al*, 1992).

The su(Hw) binding region also shares characteristics with the of the β globin LCR (Grosveld *et al*, 1987). For example, the activity of both of these regions appear to be mediated by transcription factors (Strauss *et al*, 1992; Liu *et al*, 1992; Lowrey *et al*, 1992; Talbot *et al*, 1991; Pruzina *et al*, 1991; Lavelle *et al*, 1991; Ellis *et al*, 1993;

Roseman *et al*, 1993). However, a potential difference in action is indicated by the observation that the single LCR acts to convey position-independent expression in transgenic mice (Forrester *et al*, 1990; Grosveld *et al*, 1987; Ryan *et al*, 1992; van Assendelft *et al*, 1989), whereas in studies thus far, the su(Hw) binding region needs to flank test genes in order to have the same effect (Roseman *et al*, 1993; Geyer *et al*, 1992).

5.3.4.4 SARs/ MARs as potential boundaries of chromatin domains.

It is proposed that specialized DNA sequences exist, called Scaffold-Associated Regions/ Matrix Attatchment Regions (SARs/MARs), by which the bases of the loops of the discrete and topologically independent units of gene regulation of 30 nm chromatin fibre attatch to the chromosomal scaffold (Rattner *et al*, 1985; Kellum *et al*, 1992; Ericsson *et al*, 1990; Laemmli *et al*, 1992). It is proposed that proteins interacting with these sequences could control chromatin opening and closing within the looped domain (Laemmli *et al*, 1992).

SARs were originally identified biochemically (as opposed to functionally) as DNA restriction fragments that co-purify with the nuclear- or chromosomal scaffold of restriction enzyme-digested and detergent-extracted nuclei (Laemmli *et al*, 1992; Eggert *et al*, 1991). SARs are highly A-T rich sequences (typically 70% A+T), and contain sequences similar to consensus Topoisomerase II (Topo II) recognition sequences, and Topo II is an abundant component of both nuclear matrix and chromosomal scaffold (Laemmli *et al*, 1992).

SARs have also been shown to be associated with several types of functional sequences, including Drosophila autonomously-replicating sequences (ARS), promoters, enhancers, the β globin LCR and at the boundaries of the region of DNase1 sensitivity surrounding several genes, including the A-element of the chicken lysozyme gene, which has properties of a chromatin domain boundary (Stief *et al*, 1989; Amati *et al*, 1990; Gasser *et al*, 1986; Jarman *et al*, 1988; Levy-Wilson *et al*, 1989).

Several proteins have been identified which bind selectively to SAR sequences, at least one of which is only expressed in certain tissues, and others which bind co-operatively to MAR sequences and induce DNA looping between distant sites (Dickinson *et al*, 1992; von Kries *et al*, 1991; Romig *et al*, 1992). Topo II can also

bind to SAR elements (though is not considered to have a major role in anchoring of loop bases to the chromosomal scaffold) (von Kries *et al*, 1991), as can histone H1, which binds preferentially to SARs in a highly-co-operative manner (Laemmli *et al*, 1992; Izzaurralde *et al*, 1989) (see section **5.3.5**).

Although SARs co-localize with several sequences shown to affect chromatin conformation (Stief *et al*, 1989; Amati *et al*, 1989; Gasser *et al*, 1986; Jarman *et al*, 1988; Levy-Wilson *et al*, 1989), the detection of potential SARs by detergent extraction of nuclei may be flawed (Eggert *et al*, 1991). For example, using non-detergent methods of extraction, the SAR of the Drosophila *ftz* gene is retained in the nucleus no better than a fragment which lacks an SAR, and it does not influence the local chromatin structure (Eggert *et al*, 1991). Similarly, the Drosophila 87A7 heat shock locus SAR does not function in an enhancer-blocking assay, unlike *scs* elements of the same locus (Kellum *et al*, 1992). Hence, these two "SARs", identified by biochemical means, show no evidence of being boundary elements in functional assays, and suggest that many so-called SARs may not function as domain boundaries. A possible reason why so many "SARs" co-localize with important functional elements is suggested by the fact that homeotic transcription factor protein binding sites, origins of replication, 3'UTR instability elements and SARs share "ATTA" and "ATTTA" motifs (Felsenfeld, 1992; Shaw *et al*, 1986). Thus, for example, certain A-T-rich elements may be transcription factor binding sites which are fortuitously identified as SARs due to the favouring such sites by the physical techniques used for identifying SARs.

5.3.5 Presence or absence and/or modification of histone proteins associated with genes.

5.3.5.1 Passive Role of Nucleosomes in Transcription.

In contrast to nuclear DNase1 hypersensitive sites, which lack nucleosomes (Gross *et al*, 1988), the histone content of potentially active gene loci situated in DNase1-sensitive chromatin is not grossly different from that of inactive chromatin (Felsenfeld, 1992; Clark *et al*, 1992). The major difference appears to be a slight (approximately two-fold) reduction in the amount of histone H1 present in active/ potentially active- compared to inactive chromatin (Postnikov *et al*, 1991; Kamakaka *et al*, 1990). This slight histone H1 depletion may be significant, as histone H1

proteins bind to both naked DNA and to chromatin in a highly-co-operative manner (Kamakaka *et al*, 1990; Kas *et al*, 1993), so that partial depletion of H1 in active chromatin might result in the breakdown of co-operative interactions between H1 proteins, resulting in the breakdown of the 30nm chromatin fibre (Kamakaka *et al*, 1990; Kas *et al*, 1993).

Binding of histone H1 strongly favours A-T-rich sequences, and it has been proposed that H1 proteins co-operatively bind to SAR sequences adjacent to chromatin domains, and co-operatively spread across those domains (Laemmli *et al*, 1992; Kas *et al*, 1993); the presence of an occupied boundary element, such as the β globin LCR (Forrester *et al*, 1990), adjacent to the SAR would prevent the spread of H1 into the chromatin domain, resulting in an "open" chromatin domain (Kas *et al*, 1993) (this model does not preclude H1 being bound in a non-stochiometric (non-co-operative) fashion to the "open" domain, as is observed).

Although core nucleosome and histone H1 numbers are similar in active and inactive chromatin, post-translational modification of histone proteins appears to be a significant factor in activation of chromatin (Bradbury *et al*, 1992). Histone proteins all have central globular domains, from which flexible N- and C-termini extend (Grunstein, 1990; Bradbury, 1992). The N-terminal tails of histones H3 and H4 and the C-terminal tail of histone H1 are particulaly lysine-rich , while H2A N-terminal tail is also lysine-rich to a lesser degree (Grunstein, 1990; Bradbury, 1992; Garrard, 1991). The C- and N-terminal tails of H1 and the N-terminal tail of H2B contain multiple repeats of a motif containing Ser-Pro-Pro-Lys (SPKK motif), which appears to facilitate DNA-binding (Suzuki, 1989). Notably, *all* of the sites of reversible biochemical modifications of histones, acetylation, phosphorylation and ubiquination, are located in these N- and C-terminal basic domains (Bradbury, 1992).

The very basic, nature of the core histone N-terminal tails suggests potential interaction of these domains with the phosphate backbone of DNA, or acidic domains of other protein(s), though removal of the N-termini has only minor effects on stability or assembly of nucleosomal cores (Grunsten, 1990). Acetylation of these N-terminal lysines, which neutralizes their positive charge, is accomplished by histone acetyltransferases in the nucleus (Grunstein, 1990; Cary *et al*, 1982), and potentially abolishes/ weakens interaction of the N-terminal tails with the DNA or interacting

protein(s), thus potentially deastabilising the nucleosome and facilitating access of regulatory molecules to DNA (Lee *et al*, 1993).

Increased acetylation of histones is associated with transcriptionally active/ potentially active chromatin, and appears to precede transcriptional activation (Lee *et al*, 1993; Hebbes *et al*, 1992). Thus, for example, acetylated histones are present at the DNase1 sensitive β globin locus independently of transcription of the genes therein (Hebbes *et al*, 1992). However, histone H4 acetylation is not found throughout actively transcribed chromatin, but occurs at defined sites, possibly in the non-transcribed gene-flanking regions (Turner *et al*, 1990), so that histone H4 acetylation may facilitate the binding of polymerases and other non-histone proteins to DNA prior to the onset of transcription (Turner *et al*, 1990). For example, while non-acetylated core histones prevent the binding of transcription factor TFIIIA to its binding site on DNA in model chromatin templates, acetylation of their N-terminal arms *relieves* this inhibition, and allows the TFIIIA to bind to its recognition site (Lee *et al*, 1993). This TFIIIA binding does not involve a reduction in the extent of histone-DNA interactions, and does not involve direct interaction between the nucleosomal N-terminal tails and the transcription factor, but appears to be due to a change in the path of DNA around the nucleosomes (Lee *et al*, 1993). In contrast, in yeast, promoter activation and silencing can be mediated by specific interaction between the histone H4 N-terminal tail and the appropriate DNA-bound transcription factor (Lee *et al*, 1993).

Newly-synthesised nucleosomes contain acetylated histone H4, so that half of the nucleosomes that form on newly-replicated DNA contain acetylated histones (Lee *et al*, 1993; Perry *et al*, 1991). This histone acetylation reduces H1-mediated nucleosome interactions during chromatin assembly (Perry *et al*, 1991) (which should also hold true for acetylated nucleosomal cores in active chromatin), so that nascent chromatin remains preferentially DNase1-sensitive, ie. it is unable to aggregate via H1 into the 30nm chromatin fibre (Perry *et al*, 1991).

Hyperacetylated nucleosomes containing newly-replicated DNA become progressively de-acetylated as the chromatin matures (Lee *et al*, 1993; Perry *et al*, 1991), so that the described histone acetylation might therefore provide a "window" during which transcription factors have ready access to newly-replicated DNA,

allowing the programming of gene activation (Lee *et al*, 1993; Perry *et al*, 1991). Subsequent deacetylation as the chromatin matured would allow the establishment of the 30nm fibre, and prevention of subsequent gene activation until the next round of DNA replication (Lee *et al*, 1993).

Interestingly, the glucocorticoid receptor (a SHR) recognises its binding site on non-acetylated chromatin, and in this way disrupts a positioned nucleosome on the MMTV promoter (Archer *et al*, 1991; Archer *et al*, 1992; Bruggermeier *et al*, 1991; Perlmann *et al*, 1992; Bruggermeier *et al*, 1990 and section **5.3.3.3**). However, this disruption is prevented by sodium butyrate, a short-chain fatty acid that induces histone hyperacetylation (Bresnick *et al*, 1990). This correlates with the fact that SHRs appear to be different from most other transcription factors, in that they can induce gene activation in the absence of DNA replication (Workman *et al*, 1988; Archer *et al*, 1991; Archer *et al*, 1992) but, from the above, and unlike other transcription factors, they might not be expected to induce such activation during DNA replication.

It is worth noting that acetylation may not be the only modification of histones involved in gene activation as, for example, histone H3 has recently been shown to be rapidly phosphorylated in response to growth factors, phorbol esters, okadaic acid and protein synthesis inhibitors, all of which rapidly induce early-response genes such as *c-fos* and *c-jun* (Mahadevan *et al*, 1991). Further, histone H3 in *c-fos* gene nucleosomes undergoes a rapid conformational change concommitant with gene activation (Mahadevan *et al*, 1991).

5.3.5.2 Active role of nucleosomes in transcription.

Nucleosomes can play an active as well as a passive role in transcription. As well as in the yeast system, where interaction of transcription factors with the N-terminal arm of histone H4 appears to be required for the activation and repression of certain promoters (Lee *et al*, 1993; Durrin *et al*, 1992), a nucleosome positioned at a certain site on the Xenopus vitellogenin B1 promoter is required for the stimulation of the response of the gene to estrogen by a distant upstream regulatory element (van Holde, 1993). It appears that the positioned nucleosome, by looping the DNA between the upstream estrogen response element and the promoter, allows interaction

between the estrogen receptor at the upstream element and specific transcription factor(s) bound at the promoter (van Holde, 1993).

5.3.6 DNA Methylation.

5.3.6.1 Overview.

In vertebrates, but not in lower eukaryotes, DNA can become methylated at the 5-position of cytosines of CpG dinucleotides (Bird, 1986; Simpson *et al*, 1986; Urieli-Shova *et al*, 1982) (as well as at other sites not relevant to this discussion). Between 60% and 90% of cytosines at CpGs are methylated in this way, accounting for most of the methylcytosine in the vertebrate genome (Bird, 1986), although statistically there ought to be ~5-fold greater frequency of CpG in the vertebrate genome. The paucity of CpG may be due to the fact that methylcytosine can be deaminated to give thymine which, unlike the deamination of cytosine to give uracil, is not efficiently recognised by DNA repair mechanisms, and deamination of 5-methylcytosine appears to be a major cause of somatic point mutations in the vertebrate genome (Coulondre *et al*, 1978; Jones *et al*, 1992). These points suggest that the methylation of cytosine in vertebrates must be functionally important, which is further supported by the observation that targeted mutation of the DNA methyltransferase gene in mice results in abnormal development and embryonic lethality (Li *et al*, 1992).

5.3.6.2 DNA methylation and gene activity.

About 1% of vertebrate DNA contains "islands" of DNA, usually hundreds but sometimes thousands of bps in length, in which the density of CpG dinucleotides is >10-fold higher than in the genome as a whole (Bird *et al*, 1985). In contrast to CpGs dinucleotides in the surrounding sequences, these CpG islands are completely autosomally unmethylated in both the germline and all somatic cells, and correspond to the *cis*-regulatory sequences of constitutively active housekeeping genes (Bird, 1986; Bird *et al*, 1985). Unlike housekeeping genes, most tissue-specific genes are methylated in the germ-line, throughout development and in all adult non-expressing tissues, and are only demethylated in the particular tissue in which they are normally expressed (Bird, 1986; Cedar, 1988). These observations correlate gene inactivity/ activity with methylation/ demethylation of that gene, though a fraction of tissue-

specifically expressed genes' cis-regulatory sequences are within CpG islands and are thus constitutively unmethylated (Antequera *et al*, 1990).

Further evidence for the association of non-transcribed DNA with methylation comes from study of X-chromosome inactivation in mammals. In female mammalian cells, transcriptional inactivation of one of the two X-chromosomes occurs in the embryo (Riggs *et al*, 1992). Although the two X-chromosomes are similarly methylated overall, distinct differences are observed at the single gene level. Thus, housekeeping genes such as PGK1 are actively transcribed and completely unmethylated on the active X-chromosome, but are not transcribed and completely CpG-methylated on the inactive X-chromosome in the same cell (Riggs *et al*, 1992). Treatment of cells with 5-azacytidine (5-Aza) causes heritable hypomethylation of cytosine in DNA and, specifically, demethylation and concommitant transcriptional activation of X-linked CpG island-containing housekeeping genes (Riggs *et al*, 1992; Lock *et al*, 1987). However, inactivation of the X-chromosome occurs prior to methylation of the housekeeping gene Hprt, and this and related observations suggest that methlylation is a consequence rather than a cause of gene inactivation (Lock *et al*, 1987; Buschhausen *et al*, 1987; Enver *et al*, 1988; Sullivan *et al*, 1989).

Some transcription factors are unable to bind to their recognition sites on DNA *in-vitro* if that site contains 5-methylcytosine, and this has been proposed as a potential mechanism for lack of transcription from inactive genes (Weih *et al*, 1991; Watt *et al*, 1988). However, in the case of the TAT gene, although *in-vitro* binding of the transcription factor CREB to its recognition site on DNA is prevented by methylation of that site, 5-Aza treatment of cells, which leads to demethylation of this site, does not allow cellular CREB to bind (Weih *et al*, 1991), again suggesting that chromatin context rather than methylation per se, is important for the inactivation of the gene in non-expressing cells.

Many autosomal genes in established cell lines *can* be activated by treatment of those cells with 5-Azacytidine (Gounari *et al*, 1987). However, in cell lines, many CpG islands associated with genes not directly required for cell-survival in culture become methylated, although they are in fact *un*-methylated *in-vivo* (Antequera *et al*, 1990). It is these same genes that 5-Aza treatment of cultured cell lines reactivates (Antequra *et al*, 1990), ie. 5-Aza treatment removes "unnatural" methylation but, as in

the example of the TAT gene (above), there is no evidence that 5-Aza can directly activate naturally-methylated CpG-deficient promoters (Weih *et al*, 1991; Bird, 1992).

Thus, DNA methylation may be involved in gene inactivation, but in a more subtle way than might have been first thought. Recent indentification of two proteins, MeCP1, that binds strongly *in-vitro* to DNA that contains twelve or more symmetrically-methylated CpGs in any sequence context, and MeCP2, that can bind strongly to DNA containing only a single methylated CpG dinucleotide in any sequence context, may shed light on the role played by methylation in gene repression (Boyes *et al*, 1991; Lewis *et al*, 1992).

Direct methylation-mediated repression of transcriptional activity means that methylation of the DNA recognition site for a transcription factor prevents the factor recognising this site. However, studies with MeCP1 imply it has a role as an indirect repressor of gene activity (Boyes *et al*, 1991; Boyes *et al*, 1992). Firstly, cells and cell extracts containing low levels of MeCP1 cannot efficiently repress transcription of methylated genes (Boyes *et al*, 1991). Secondly, seven fully-methylated promoters were functionally tested *in-vitro* and *in-vivo*: in the absence of MeCP1, methylation-mediated repression of these promoters was weak, but when MeCP1 was present, no transcription was detected, so that indirect inhibition of promoter activity by MeCP1 binding appears to be more important than direct inhibition (Boyes *et al*, 1991; Boyes *et al*, 1992).

Interestingly, in transfection studies, sparse methylation can repress activity of a weak promoter, which is overcome by the presence in *cis* of an enhancer, while a densely-methylated promoter is not active and cannot be activated by an enhancer (Boyes *et al*, 1992). MeCP1 can bind weakly to sparsely-methylated sequences and strongly to densely-methylated sequences (Boyes *et al*, 1991; Boyes *et al*, 1992), and in the above experiments, when MeCP1 was absent, methylation had minimal effect on transcription. Hence MeCP1 may play a role, further described below, in the repression of methylated genes.

5.3.7 Model for gene expresssion in chromatin.

5.3.7.1 How active genes stay active and inactive genes remain inactive.

It is evident that there are several differences between active and inactive genes which may contribute to why in a particular cell-type one tissue-specific gene may remain permanently "on", while another tissue-specific gene, not expressed in that cell-type, remains permanently "off". Firstly, active genes replicate early in S-phase, while inactive genes generally replicate late in S-phase, and chromosomal context, rather than differences in origins of replication between early- and late-replicating genes, appears to determine the time of replication of a gene (Fangman *et al*, 1992; Braunstein *et al*, 1982; Forrester *et al*, 1991). This can be explained by the fact that inactive genes are in more condensed chromatin than active genes, wherein the presence of transcription factor bound to DNA increases the accessibility of replication origin(s) situated on the nearby DNA (Forrester *et al*, 1990; Cheng *et al*, 1989). If certain transcription factors are present in the cell in limiting amounts, the first set of binding sites to replicate would be expected to sequester most of the available transcription factor (Hatton *et al*, 1988). Hence, this is a mechanism for perpetuating the "on" status of one set of genes and the "off" status of another set of genes.

A second mechanism for perpetuating the activity status of a gene may be DNA methylation. As described, a protein that binds to symmetrically-methylated CpGs, MeCP1, has been strongly implicated in the indirect repression of transcription from methylated promoters (Boyes *et al*, 1991; Boyes *et al*, 1992). It is tempting to believe that MeCP1, or a protein with similar characteristics, is involved in the repression of binding of transcription factors to DNA during DNA replication. Although MeCP1 binds to symmetrically-methylated DNA, and DNA replication results in the formation of hemi-methylated DNA, it is evident that this DNA is rapidly fully methylated by DNA methyltransferase at the replication fork (Leonhardt *et al*, 1992). It is evident that MeCP1 can compete with transcription factors for DNA binding (Boyes *et al*, 1991; Boyes *et al*, 1992). Hence, MeCP1 would compete with transcription factors for binding to newly-replicated symmetrically-methylated DNA, while the DNA of active genes, being unmethylated, would be accessible to transcription factors, but would not be recognised by MeCP1 (Boyes *et al*, 1991; Boyes *et al*, 1992). As DNA binding of certain transcription factors is strongly reduced if the binding site contains even *hemi*-methylated CpG (Weih *et al*, 1991),

this would combine with the above mechanism to further ensure that newly-replicated DNA of active genes would be strongly favoured over that of inactive genes in terms of transcription factor binding.

It has recently been demonstrated that histone H1 binds preferentially to symmetrically-methylated DNA compared to non-specific DNA (Jost *et al*, 1992). Because of the fully methylated status of inactive genes, binding of histone H1 to such regions is therefore favoured, allowing incorporation of that DNA into a more condensed higher-order structure, and ensuring its late replication (see above).

5.3.7.2 How inactive genes become active.

Nucleosomes formed on newly-replicated DNA are acetylated (Hebbes *et al*, 1992) and, as discussed, acetylated nucleosomes, in contrast to the non-acetylated nucleosomes of mature chromatin, allow non-steroid hormone receptor transcription factor binding to the DNA complexed with them. Hence, although there is rapid deposition of nucleosomes onto newly-replicated DNA behind the replication fork (Svaren *et al*, 1990), the acetylation of the newly-deposited nucleosomes may provide a "window" of opportunity for transcription factor binding to the DNA. Such transcription factor binding may be aided by the fact that there are at least 200 bps of DNA behind the replication fork that have not yet become bound by nucleosomes (Svaren *et al*, 1990).

It is evident that for an inactive gene to become active, the transcription factors available for binding to its regulatory elements must not be limiting at the time the gene replicates, and the binding of transcription factors to the regulatory elements must be strong enough to compete out any MeCP1-like factor(s) bound to CpG dinucleotides within the regulatory elements.

5.3.8 Model for transcription on a chromatin template.

5.3.8.1 Initiation.

Regulation of transcription is generally at the level of initiation. As described, active chromatin is slightly depleted in histone H1, but contains levels of nucleosomal cores similar to those present in inactive chromatin (Felsenfeld, 1992; Clark *et al*, 1992). However, those histone cores flanking the coding sequence are modified by acetylation, and this may allow access to the DNA of regulatory transcription factors

and the formation of the transcriptional initiation complex (Cary *et al*, 1982; Turner *et al*, 1991; Lee *et al*, 1993; Hebbes *et al*, 1992; Turner *et al*, 1990).

5.3.8.2 Elongation.

It appears that RNA polymerase displaces histone cores from the DNA in front of it, and that these histones reform on the nearest available non-histone-bound DNA, which is behind the transcribing RNA polymerase (Clark *et al*, 1992; Morse, 1992). The DNA around nucleosomes is negatively supercoiled, and nucleosomes form on negatively-supercoiled- in preference to positively-supercoiled DNA (Clark *et al*, 1992; Morse, 1992). Transcribing RNA polymerase apparently creates a wave of positive supercoiling ahead of it, and a local domain of negative supercoiling behind it , which may induce the displacement of histone cores from in front of the RNA polymerase and deposition behind it, as is observed (Clark *et al*, 1992; Morse, 1992).

5.4 Experimental aims.

Prior to this study, MIP1α had been identified in two ways: a) as an inhibitor of the proliferation of haemopoietic stem cells, produced by a population of bone marrow macrophages and present in normal bone marrow; b) as a haemopoietic cell-specific gene rapidly induced in response to inflammatory- or immunomodulatory stimuli. Furthermore, MIP1α had been identified as a gene constitutively expressed at a high level in haemopoietic fresh tumour cells, and such disregulated expression of MIP1α in the bone marrow could have profound effects on the haemopoietic system. However, the molecular mechanisms behind each of these observations had not been investigated. As gene expression is ultimately controlled at the level of transcription, and because macrophages were identified as the cells that produce MIP1α in normal bone marrow, the aim of this thesis was therefore to examine the transcriptional regulation of the MIP1α gene in macrophages in comparison to non-expressing cell types.

As a genomic clone putatively containing the MIP1α gene had been isolated in this laboratory immediately prior to this thesis, my initial aim was to confirm the presence of the MIP1α gene in this clone and sequence the gene. The aim was then to characterize the transcriptional regulatory elements and transcription factors

conferring macrophage-specific expression on the MIP1α gene in unstimulated cells and in response to a known inducer of MIP1α, lipopolysaccharide. As many of the same transcriptional regulatory regions (though not necessarily the same transcription factors) are used to express a particular gene in different expressing cell-types and in response to different cell stimuli, it was hoped that these studies would identify potential inducers of MIP1α gene expression in both macrophages and other cell types. Furthermore, it was hoped that these studies would identify potential mechanisms by which the expression of the MIP1α gene could be disregulated at the level of transcription.

PART 2: MATERIALS AND METHODS.

CHAPTER 6: MATERIALS.

Table 6.1

pUC18 : the following plasmids are based on the pUC18 plasmid, which was purchased from USB, Cleveland, Ohio, USA.

Orientation Name	Identity	Size(bp)	mcs sites	5'	3'
p18E7	MIP1a locus EcoR1 fragment	~4400	EcoR1	Sst1	EcoR1
p18SL3	MIP1a locus Sst1 fragment	~1800	Sst1	EcoR1	Kpn1
p18S2	p18E7-derived Sst1 fragment	~1400	Sst1	Kpn1	EcoR1
p18L4	MIP1a locus HincII fragment	~1150	HincII	Pst1	Xba1
p18HII	p18E7-derived HincII fragment	~750	HincII	?	?
p18N4	MIP1a promoter Nco1 fragment	~550	HincII	Xba1	Pst1
p18SL3NS	p18SL3-derived Sst1-Nco1 fragment	~850	(Sst1)	Sst1	BamH1
p18LS2Acc	p18S2-derived Acc1 fragment	~1200	Acc1?		?
p18SS2Acc	p18S2-derived Sst1-Acc1 fragment	~150	(Sst1)	Kpn1	EcoR1
p18NE1	p18SL3-derived Sst1-Nco1 fragment	~350	(Sst1)	(EcoR1)	Kpn1
p18M-900	MIP1a promoter -900 to +36 bp fragment PCR-derived from p18E7, using primers PCR4 and PCR5.	~940	HindIII/ BamH1	HindIII	BamH1
p18M-270	MIP1a promoter -270 to +36 bp fragment PCR-derived from p18E7, using primers PCR2 and PCR3.	~300	EcoR1/ Xba1	EcoR1	Xba1
p18M-160	MIP1a promoter -160 to +36 bp fragment derived from p18M-900 .	~200	(HincII)/ BamH1	Pst1	BamH1
p18M-350	MIP1a promoter -350 to +36 bp fragment derived from p18M-900.	~390	(HincII)/ BamH1	Pst1	BamH1
p18M-470	MIP1a promoter -470 to +36 bp fragment derived from p18M-900.	~510	(HincII)/ BamH1	Pst1	BamH1
p18MNR1	p18E7-derived EcoRV-Sst1 fragment	~550	(HincII)/ Sst1	Pst1	Sst1
p18MNR2	p18E7-derived Sst1-HincII fragment	~320	Sst1/ HincII	Sst1	HincII
p18MNR3	p18E7-derived HincII-Xma1 fragment	~610	Sma1	BamH1	Sst1
p18HGHNR2	p0GH-derived Sma1-Sma1 fragment	~670	Sma1	?	?

pBluescript SK(-): the following plasmids are based on the pBluescript SK(-) (pSK-) plasmid, obtained from Stratagene, La Jolla, California.

Name	Identity	Size(bp)	PCR primers used	mcs sites	Orientation 5'	Orientation 3'	Comments.
pSK-MIP1α	MIP1a gene -220 to +156 fragment generated by PCR using p18M-470 as template	376	MIP-ee1, pM-220p	HindIII/ Xba1	HindIII	Xba1	———
pSK-MIPcDNA	MIP1a cDNA	~800	?	?	Xba1	?	Obtained from Dr. M.Plumb.

pSPT18: the following plasmid is based on the pSPT18 plasmid, obtained from Boehringer-Mannheim.

Name	Identity	Size (bp)	mcs sites	5'	3'
pSPT18MIP1α/ hGH	pGHM-350-derived HincII-Ball fragment	~500	Sma1	Xba1	Sst1

Table 6.2

p0GH: the following plasmids are based on the p0GH plasmid, which was a gift from Dr. M.Plumb.

All subclones in p0GH are either wild-type or mutant MIP1a promoter deletion fragments, and are all oriented with either the p0GH BamH1 or Xba1 site (as indicated) at their 3' ends. All mutant MIP1a promoters were derived by PCR using the indicated primers, and linearized (using PvuII) p18M-350 or p18M-470 plasmid as template.

Name	Identity	Size(bp)	PCR primers used	p0GH sites
pGHM-900	MIP1a promoter -900 to +36 bp fragment	~940	----------	HindIII/BamH1
pGHM-270	MIP1a promoter -270 to +36 bp fragment	~300	----------	(HincII)/Xba1
pGHM-160	MIP1a promoter -160 to +36 bp fragment	~200	----------	HindIII/BamH1
pGHM-160am41	MIP1a mutant promoter -160 to +36 bp derived from pGHM-470am41	~200	----------	(HincII/BamH1)
pGHM-160am44	MIP1a mutant promoter -160 to +36 bp derived from pGHM-470am44	~200	----------	(HincII/BamH1)
pGHM-190	MIP1a promoter -190 to +36 bp fragment	~230	pM-190p, PCR5	HindIII/BamH1
pGHM-190em1	MIP1a mutant promoter -190 to +36 bp,	~230	pM-190em1p, PCR5	HindIII/BamH1
pGHM-190em2	MIP1a mutant promoter -190 to +36 bp	~230	pM-190em2p, PCR5	HindIII/BamH1
pGHM-190em3	MIP1a mutant promoter -190 to +36 bp	~230	pM-190em3p, PCR5	HindIII/BamH1
pGHM-190em4	MIP1a mutant promoter -190 to +36 bp	~230	pM-190em4p, PCR5	HindIII/BamH1
pGHM-190em5	MIP1a mutant promoter -190 to +36 bp	~230	pM-190em5p, PCR5	HindIII/BamH1
pGHM-350	MIP1a promoter -350 to +36 bp fragment	~390	----------	HindIII/BamH1
pGHM-350am40	MIP1a mutant promoter -350 to +36 bp	~390	FP-am40pa, FP-am40pb, LUP,LRP	HindIII/BamH1
pGHM-350am41	MIP1a mutant promoter -350 to +36 bp	~390	FP-am41p, FP-am41pa, LUP,LRP	HindIII/BamH1
pGHM-350am44	MIP1a mutant promoter -350 to +36 bp	~390	FP-am44p, FP-am44pa, LUP, LRP	HindIII/BamH1
pGHM-350am46	MIP1a mutant promoter -350 to +36 bp	~390	FP-am46pa, FP-am46pb LUP, LRP	HindIII/BamH1
pGHM-350am47	MIP1a mutant promoter -350 to +36 bp	~390	FP-am47pa, FP-am47pb, LUP, LRP	HindIII/BamH1
pGHM-350am48	MIP1a mutant promoter -350 to +36 bp	~390	FP-am48pa, FP-am48pb LUP, LRP	HindIII/BamH1
pGHM-350bm30	MIP1a mutant promoter -350 to +36 bp	~390	FP-bm30pa, FP-bm30pb, LUP, LRP	HindIII/BamH1
pGHM-350bm35	MIP1a mutant promoter -350 to +36 bp	~390	FP-bm35pa, FP-bm35pb, LUP, LRP	HindIII.BamH1
pGHM-350pcm10	MIP1a mutant promoter -350 to +36 bp	~390	pFP-cm10pa, pFP-cm10pb, LUP, LRP	HindIII/BamH1
pGHM-350dcm20	MIP1a mutant promoter -350 to +36 bp	~390	dFP-cm20pa, dFP-cm20pb, LUP, LRP	HindIII/BamH1
pGHM-350dm1	MIP1a mutant promoter -350 to +36 bp	~390	FP-dm1pa, FP-dm1pb, LUP, LRP	HindIII/BamH1
pGHM-350dm2	MIP1a mutant promoter -350 to +36 bp	~390	FP-dm2pa, FP-dm2pb, LUP, LRP	HindIII/BamH1
pGHM-350dm3	MIP1a mutant promoter -350 to +36 bp	~390	FP-dm3pa, FP-dm3pb, LUP, LRP	HindIII/BamH1
pGHM-350dm4	MIP1a mutant promoter -350 to +36 bp	~390	FP-dm4pa, FP-dm4pb, LUP, LRP	HindIII/BamH1
pGHM-350em3	MIP1a mutant promoter -350 to +36 bp	~390	FP-em3pa, FP-em3pb, LUP, LRP	HindIII/BamH1
pGHM-350em4	MIP1a mutant promoter -350 to +36 bp	~390	FP-em4pa, FP-em4pb, LUP, LRP	HindIII/BamH1
pGHM-470	MIP1a promoter -470 to +36 bp fragment	~510	----------	HindIII/BamH1
pGHM-470am41	MIP1a mutant promoter -470 to +36 bp	~510	FP-am41p, FP-am41pa LUP, LRP	HindIII/BamH1
pGHM-470am44	MIP1a mutant promoter -470 to +36 bp	~510	FP-am44p, FP-am44pa LUP, LRP	HindIII/BamH1
pGHM-470bm35	MIP1a mutant promoter -470 to +36 bp	~510	FP-bm35pa, FP-bm35pb, LUP, LRP	HindIII/BamH1

Table 6.3

Sequencing primers

Name	Sequence	Source
M13 Reverse	5' AACAGCTATGACCATG	USB
M13 Universal	5' TAAAACGACGGCCAGT	USB
LRP	5' CAGGAAACAGCTATGACCATGATTACGAATTCG	Beatson Institute
LUP	5' CCCAGTCACGACGTTGTAAAACGACGGCCAG	Beatson Institute
p0G1	5' CATTGCAGCTAGGTGAGCGTCCACAG	Beatson Institute
MOL-1	5' GCAGCCATACTGGTTA	Beatson Institute
MOL-2	5' TGACATTTCTGGTCCCAG	Beatson Institute
MOL-3	5' GCTGCTTCTCCTACAGCC	Beatson Institute
MOL-4	5' TGGTCTGCCGGAGACC	Beatson Institute
MOL-5	5' TCCAATTAAGGCCGGAA	Beatson Institute
MOL-6	5' ATTATGAAGTCGGAGAAG	Beatson Institute
MOL-7	5' GAACCACAGAGGAAGTCA	Beatson Institute
5498	5' CAGAATTCTCAGGCAATCAGTTCCAGG	Dr. S.Wolpe.
5496	5' AGCTCGAGTCATGAAGGTCTCCACCAC	Dr. S.Wolpe

Oligonucleotides for EMSAs

Name	Sequence	Source
FP-am44	5' CCCGAATTCCAATTACACAACTGCTAGG	Beatson Institute
FP-am41	5' CCCGAATTCCATTTCCTCTTTTGCTAGG	Beatson Institute
FP-a	5' CCCAGATTCCATTTCCTCATCTGCTAGG	Beatson Institute
PU.1	5' GATCCATAACCTCTGAAAGAGGAACTTGGTTAGGT	Dr.K. McLeod
PEA3	5' GATCCTCGAACTTCCTGCTCGAA	Dr.K.McLeod
hG-CSF	5' GGATCAGAGATTCCACAATTTC	Beatson Institute
KBpd	5' CAACGGCAGGGGAATTCCCCTCTCCTT	Beatson Institute
FP-b	5' CTTAAAATTTTCCCTCCTCAC	Beatson Institute
pFP-c	5' TCCATCATGACACCATTGCTGTG	Beatson Institute
dFP-c	5' CCCTGGGTTGTGTAATATCC	Beatson Institute
FP-bm35	5' CTTAAAATTCTAGATCTAGAG	Beatson Institute
mAP1	5' CCGAAGTTCAGATTCAGAACTCAGGG	Beatson Institute
FP-(d+e)	5' AGACTTATATCTCAGAGATGCTATTCTTAGATATCCTGGGCCCCTGTGGTCACTGTGGACCCTGG	Beatson Institute
FP-d	5' GGGCCCCTGTGGTCACTGTGGACCCTGG	Beatson Institute
mGPE-1	5' ACAGAGATTCCCCGATTTCACAAAAACTTTCGC	Beatson Institute
E3-AP-1	5' CCGAAGTTCAGATGACTAACTCAGGG	Dr. A.Pintzas
C/EBP	5' GCTGCAGATTGCGCAATCTGCAGC	Dr. A.Pintzas
cep3a	5' GATCCAAACCAGCCAATGAGAACTGCTCCA	Dr.M.Pintzas
FP-e	5' GACTTATATCTCAGAGATGCTATTCTTAGATATCCTGGG	Beatson Institute
FP-f	5' AAGAAGAAAGAATAGGTCACGAGTTGAGAGCTGAGACTTATAT	Beatson Institute

Bromodeoxyuridine (U)-substituted oligonucleotides for UV crosslinking studies.

Name	Sequence	Source
FP-aUa	CCCAGATTCCAUUUCCUCAUCUGCTAGG	Beatson Institute
FP-aUb	CCTAGCAGAUGAGGAAAUGGAATCTGGG	Beatson Institute

Table 6.4

PCR primers

Name	Sequence	Source
LRP	5'CAGGAAACAGCTATGACCATGATTACGAATTCG	Beatson Institute
LUP	5' CCCAGTCACGACGTTGTAAAACGACGGCCAG	Beatson Institute
PCR1	5' GGGGAATTCTTCACTTTGTAAAAC	Beatson Institute
PCR2	5' GGGGAATTCATAAGAGAAACTACTT	Beatson Institute
PCR3	5' CCCTCTAGAGCAGAACAGAAAAGGGACTGGTA	Beatson Institute
PCR4	5' GGGAAGCTTCACTTTGTAAAACTTG	Beatson Institute
PCR5	5' GGGGGATCCAAAGGGACTGGTACTCGCTGCTGCTT	Beatson Institute
MIP-eel	5' GCGCGCTCTAGAATGGCGCTGAGAAGACTTGGTTGCAG	Beatson Institute
HGH-eel	5' GGGGGCCTAGGCTGTAGCCATTGCAGCTAGGTGAGCG	Beatson Institute
FP-em41p	5' CGAATTCCATTTCCTCTTTTGCTAGGGCTACC	Beatson Institute
FP-em41pa	5' GGTAGCCCTAGCAAAAGAGGAAATGGAATTCGGGGCTGAGGAGGG	Beatson Institute
FP-em44p	5' CCGAATTCCAATTACACAACTGCTAGGGCTACC	Beatson Institute
FP-em44pa	5' GGTAGCCCTAGCAGTTGTGTAATTGGAATTCGGGGCTGAGGAGGG	Beatson Institute
FP-bm35pa	5' CTAGATCTAGAGCCCCAGATTCCATTTCC	Beatson Institute
FP-bm35pb	5' GGAAATGGAATCTGGGGCTCTAGATCTAGAATTTTAAGCACAGC	Beatson Institute
pM-190p	5' GCGCGCAAGCTTAGACTTATATCTCAGAGATGCTATTC	Beatson Institute
pM-220p	5' GCGCGCAAGCTTAAGAAAGAATAGGTCACGAGTTGAG	Beatson Institute
pM-190em1p	5' GCGCGCAAGCTTGAATTCATATCTCAGAGATGCTATTC	Beatson Institute
pM-190em2p	5' GCGCGCAAGCTTAGACTTGAATTCCAGAGATGCTATTCTTAG	Beatson Institute
pM-190em3p	5' GCGCGCAAGCTTAGACTTATATCTGAATTCTGCTATTCTTAGATATCC	Beatson Institute
pM-190em4p	5' GCGCGCAAGCTTAGACTTATATCTCAGAGAGAATTCTCTTAGATATCCTGGGCC	Beatson Institute
pM-190em5p	5' GCGCGCAAGCTTAGACTTATATCTCAGAGATGCTATGAATTCATATCCTGGGCCCCTGTG	Beatson Institute
FP-bm30pa	5' GCTGTGCTTAAAATGAGCTCTCCTCACCCCCAGATTC	Beatson Institute
FP-bm30pb	5' GGGGTGAGGAGAGCTCATTTTAAGCACAGCAATG	Beatson Institute
pFP-em10pa	5' GTAATATCCATCATGACACGAATTCTGTGCTTAAAATTTTCC	Beatson Institute
pFP-em10pb	5' GCACAGAATTCGTGTCATGATGGATATTAC	Beatson Institute
dFP-em20pa	5' GTGGACCCTGAGCTCTGTGAGCTCCATCATGACACC	Beatson Institute
dFP-em20pb	5' GTCATGATGGAGCTCACAGAGCTCAGGGTCCACAGTGAC	Beatson Institute
FP-em40pa	5' CACCCCCAGAATTCATGAATTCATCTGCTAGGGCTAC	Beatson Institute
FP-em40pb	5' CTAGCAGATGAATTCATGAATTCTGGGGGTGAGGAG	Beatson Institute
FP-em46pa	5' CCATTTCCACATCTGCTCGAGCTACCTATAAGAG	Beatson Institute
FP-em46pb	5' CTTATAGGTAGCTCGAGCAGATGTGGAAATGGAATCTGGG	Beatson Institute
FP-em47pa	5' GATTCCATTTCCTCTTTTGCTCGAGCTAGCTATAAGAG	Beatson Institute
FP-em47pb	5'GGTAGCTCGAGCAAAAGAGGAAATGGAATCTG	Beatson Institute
FP-em48pa	5' CCTCACCCCCGAGCTCCATTTCCTCATCTGCTAGG	Beatson Institute
FP-em48pb	5' CAGATGAGGAAATGGAGCTCGGGGGTGAGGAGGG	Beatson Institute
FP-dm1pa	5' GATATCCTGAATTCTTGTGGTCACTGTGGACCCTG	Beatson Institute
FP-dm1pb	5' CCACAGTGACCACAAGAATTCAGGATATCTAAGAATAG	Beatson Institute
FP-dm2pa	5' CCTGGGCCCGAATTCTCACTGTGGACCCTGGG	Beatson Institute
FP-dm2pb	5' CAGGGTCCACAGTGAGAATTCGGGCCCAGGATATC	Beatson Institute
FP-dm3pa	5' GATATCCTGGGCCCCTGTGAGAATTCTGGACCCTGGGTTGTG	Beatson Institute
FP-dm3pb	5' CCAGGGTCCAGAATTCTCACAGGGGCCCAGGATATC	Beatson Institute
FP-dm4pa	5' CCCCTGTGGTCACTAGAATTCCTGGGTTGTGTAATATCC	Beatson Institute
FP-dm4pb	5' CACAACCCAGGAATTCTAGTGACCACAGGGGC	Beatson Institute
FP-em3pa	5' GACTTATATCTGAATTCTGCTATTCTTAGATATCC	Beatson Institute
FP-em3pb	5' CTAAGAATAGCAGAATTCAGATATAAGTCTCAGC	Beatson Institute
FP-em4pa	5' CTTATATCTCAGAGAGAATTCTCTTAGATATCCTGG	Beatson Institute
FP-em4pb	5' GATATCTAAGAGAATTCTCTCTGAGATATAAGTCTC	Beatson Institute

6.5 Tissue Culture

6.5.1 Cell-lines.

All cell lines used in this study were obtained from laboratory stocks.

6.5.2 Tissue culture supplies.

Supplier	Address	Product
Beatson Institute Central Services	Beatson Institute	Penicillin (7.5 mg/ml) Streptomycin (10 mg/ml) Sterile CT buffer Sterile PBS Sterile glassware and pipettes
GIBCO Europe Life Technologies Ltd.	Paisley, Scotland	Special Liquid Medium OPTIMEM 1 Medium Foetal calf serum 2.5% (w/v) trypsin 200 mM glutamine Lipofectin reagent
Costar	MA 02139, USA	96 well cell culture cluster Cell scrapers
A/S Nunc	Roskilde, Denmark	Tissue culture flasks Nunc cryotubes
Becton Dickinson, U.K.Ltd.	Plymouth, PL6 7BP	Tissue culture plates (35 mm, 60 mm, 9 cm)
DIFCO Laboratories	Detroit, Michigan, USA	Lipopolysaccharide W S. Typhimurium (100 mg)
SIGMA Chemical Co. Ltd.	Poole, Dorset.	Mouse α and β fibroblast interferon (I 1258)
Genzyme Corporation	West Malling, Kent, ME19 6HG	Recombinant mouse interferon γ
British Biotechnology Ltd.	Abingdon, Oxon, OX14 3YS	Recombinant human TGF beta 1

6.6 Bacterial hosts and media.

Supplier	Address	Product
S.Lowe	Beatson Institute	*E.Coli* host strain JM83
GIBCO Europe Life Technologies Ltd.	Paisley, Scotland	*E.Coli* host strain DH5α Ampicillin
Beatson Institute Central Services	Beatson Institute	L-broth Sterile glassware
DIFCO Laboratories	Detroit, Michigan, USA	Agar Bacto-tryptone
BDH Chemicals, Ltd.	Poole, Dorset	Super-broth components
Beta Laboratories	East Molesey, Surrey	Yeast extract

Bibby-Sterilin Ltd.	Stone, Staffordshire	Sterilin 9 cm bacteriological plates

6.7 Plasmids and bacteriophages

Supplier	Address	Product
GIBCO EuropeLife Technologies Ltd.	Paisley, Scotland	pUC18 plasmid pUC19 plasmid
Stratagene Ltd.	Cambridge, England	pBluescript II SK(-) phagemid
Dr. M.Plumb	Oxford, England	p0GH plasmid pTKGH plasmid pSK(-) MIP1α cDNA plasmid
Dr. D.Wallace, Glaxo Group Research, Ltd.	Greenford, U.K.	pSV2APAP plasmid
M.Walker	Beatson Institute	pCRII plasmid (Invitrogen Corporation),containing rat glyceraldehyde - 3-phosphate dehydrogenase (GAPDH) cDNA containing sequences between bp 300 and 1020.
Boehringer-Mannheim UK	Lewes, East Sussex	pSPT18 plasmid
Clonetech Laboratories	USA	λ EMBL3 murine (DBA/2J) adult liver genomic library

6.8 Kits.

Supplier	Address	Product
Boehringer-Mannheim UK	Lewes, East Sussex	Random-primed DNA labelling kit SP6/ T7 transcription kit
AMS Biotechnology UK Ltd.	Witney, Oxon, OX8 7GE	RPA II ribonuclease protection assay kit
United States Biochemical	Cleveland, Ohio, USA	Sequenase version 2.0 DNA sequencing kit
Qiagen Inc.	Chatsworth, CA 91311, USA	Qiagen plasmid preparation kits
Nichols Institute Diagnostics	Newport, Saffron Walden, Essex CB11 3PQ	HGH 100T human growth hormone radioimmunoassay kit
Du Pont International		Maxam-Gilbert sequencing reagents kit

6.9 Membranes, paper and X-ray film.

Supplier	Address	Product

Amersham International plc.	Amersham, Bucks.	Hybond N nylon membranes Hybond C nitrocellulose membranes
Bethesda Research Laboratories, GIBCO, Ltd.	Paisley, Scotland	Dialysis tubing
Vernon-Carus, Ltd.	Preston, Lancashire	Gauze swabs
Whatman International Ltd.	Maidstone, Kent	3MM filter paper GF-C glass microfibre filters
Eastman Kodak Co.	Rochester, NY, USA	X-OMAT AR X-ray film DUP-1 duplicating film
Presentation Technology, Ltd.	Clydebank, Scotland	P1-3 and P1-4 AGFA Rapitone paper
Technical Photo Systems	Cumbernauld, Scotland	Fuji RX Medical X-ray film

6.10 Nucleotides, polynucleotides and DNA.

Supplier	Address	Product
Amersham International, plc	Amersham, Bucks.	[α-^{32}P]-dCTP: 3000 Ci/ mmol [α-^{35}S]-dATP: 1000 Ci/ mmol [α-^{32}P]-UTP: 800 Ci/ mmol [γ-^{32}P]-dATP: 5000 Ci/ mmol
Boehringer-Mannheim	Lewes, East Sussex	GTP,ATP, CTP (100 mM lithium salts)
Pharamacia LKB Biotechnology	Milwaukee, Wisconsin, USA	dNTPs (100 mM set) Poly (dI-dC).poly (dI.dC)
SIGMA Chemical Co., Ltd.	Poole, Dorset	Salmon sperm DNA
Bethesda Research Laboratories, GIBCO, Ltd.	Paisley, Scotland	DNA molecular weight markers: 1 kbp ladder, bacteriophage φX-174 DNA (Hae III digested), bacteriophage λ DNA (Hind III-digested) Yeast tRNA

6.11 Gels and columns.

Supplier	Address	Product
Bethesda Research Laboratories, GIBCO, Ltd.	Paisley, Scotland	Agarose and Low Melting-Point agarose
Severn Biotech., Ltd.	Kidderminster	Design-a-Gel 40% (w/v) acrylamide, 2% (w/v) bis-acrylamide solution
BDH Chemicals, Ltd	Poole, Dorset	Acrylamide bis-acrylamide

Schleicher and Schuell	Dassel, Germany	Elutip-D-columns
Bio-Rad Laboratories, Ltd.	Watford, U.K.	Bio-Gel A50M agarose beads

6.12 Enzymes, enzyme buffers and enzyme inhibitors.

Supplier	Address	Product
Bethesda Research Laboratories, GIBCO, Ltd.	Paisley, Scotland	Restriction endonucleases, with buffer concentrates Taq DNA polymerase (10 U/ µl) plus 10x PCR reaction buffer T4 DNA ligase (1 U/ µl) plus 5x ligation buffer Proteinase K Calf intestinal alkaline phosphatase (24 U/ µl)
Northumbria Biologicals Ltd.	Cramlington, Northumberland	T4 polynucleotide kinase (10 U/ µl) Klenow DNA polymerase (1 U/ µl) Restriction endonucleases, with buffer concentrates
Boehringer-Mannheim UK	Lewes, East Sussex	RNase A DNase1
Pharmacia LKB Biotechnology	Milwaukee, Wisconsin, USA	One-phor-all plus restriction enzyme buffer concentrate.
SIGMA Chemical Co., Ltd.	Poole, Dorset	Diethylpyrocarbonate (DEPC) Lysozyme β-glycerophospate Sodium orthovanadate Phenylmethylsulphonyl fluoride (PMSF) Benzamidine Sodium butyrate Levamisole Leupeptin Aprotinin Bestatin Pepstatin A Creatine kinase

6.13 Chemicals.

Supplier	Address	Product
SIGMA Chemical Co., Ltd.	Poole, Dorset	Bromophenol blue Dithiothreitol (DTT) MOPS Spermidine TEMED Triton X-100 Diethanolamine SIGMA 104 phosphatase substrate Creatine phosphate L-glutamic acid

James Burrough, Ltd.	Witham, Essex	Ethanol
Rathburn Chemicals, Ltd.	Walkerburn, Scotland	Water-saturated phenol
Cinna/ Biotecx Laboratories, Inc.	Houston, Texas, USA	RNazol B
Northumbria Biologicals, Ltd.	Cramlington, Northumberland	Bovine serum albumin (20% (w/v))
Fisons Scientific Equipment	Loughborough	Formaldehyde (38% (w/v))
Fluka Chemika-Biochemika AG	Buchs, Switzerland	Formamide
BDH Chemicals Ltd.	Poole, Dorset	All chemicals used that are not listed above

6.14 Solutions.

Solution	Solution components
50x TAE	2 M Tris-acetate 0.05 M EDTA (pH 8.0)
10x TBE	0.9 M Tris-borate 0.02 M EDTA (pH 8.0)
10x TE (pH X)	100 mM Tris.Cl (pH X) 10 mM EDTA (pH 8.0)
20x SSPE	3 M Sodium chloride 180 mM Sodium dihydrogen orthophosphate 20 mM EDTA
20x SSC	3 M Sodium chloride 0.3 M Sodium citrate
2x Storage Buffer (SB)	100 mM Sodium chloride 40 mM Hepes (pH 7.9) 10 mM Magnesium chloride 0.2 mM EDTA 40% (v/v) glycerol
2x TMS	0.5 M Sucrose 10 mM Magnesium chloride 200 mM Tris-Cl [pH 7.5]
E_{50} buffer	50 mM Ammonium sulphate 20 mM Hepes [pH 7.9] 5 mM Magnesium chloride 0.1 mM EDTA 0.1% (v/v) Brij-35 20% glycerol
2x Hypotonic buffer	20 mM Tris-Cl [pH 7.5] 20 mM Sodium chloride

	3.2 mM Magnesium chloride 2 mM Calcium chloride
Glutamate buffer	0.125 M Potassium glutamate 10 mM Hepes [pH 8.0] 5 mM Magnesium chloride 1 mM EGTA 2 mM DTT 40% (v/v) glycerol
2x HBS	0.28 M Sodium chloride 10 mM Potassium chloride 1.5 mM Disodium orthophosphate 12 mM Dextrose 50 mM Hepes
Glucose buffer	50 mM glucose 25 mM Tris-Cl [pH 8.0] 10 mM EDTA
Proteinase K buffer	10 mM Tris-Cl [pH 7.8] 5 mM EDTA 0.5% SDS
DEA buffer	1 M Diethanolamine 0.28 M Sodium chloride 0.5 mM Magnesium chloride pH to 9.85 with concentrated hydrochloric acid.
10x MOPS buffer (pH 7.0)	0.4 M MOPS (pH 7.0) 0.1 M Sodium acetate 10 mM EDTA
50x Denhardt's solution	1% (w/v) Ficoll-400 1% (w/v) Polyvinylpyrrolidone 1% (w/v) Bovine serum albumin (fraction V)
Northern/ Southern (pre-) hybridization solution	5x SSPE 5x Denhardt's 0.5% (w/v) SDS 50% (v/v) Formamide 10 µg/ml Sonicated salmon sperm DNA 1 µg/ml poly A 1 µg/ml poly C
High salt buffer (HSB)	1.0 M Sodium chloride 20 mM Tris-Cl [pH 7.5] 1 mM EDTA [pH 8.0]
Low salt buffer (LSB)	0.2 M Sodium chloride 20 mM Tris-Cl [pH 7.5] 1 mM EDTA [pH 8.0]

CHAPTER 7: METHODS.

7.1 TISSUE CULTURE.

7.1.1 Cell culture.

Mouse monocyte/macrophage cell-lines RAW 264.7 and J774.2, derived from an Abelson murine leukemia virus (A-MuLV)-induced tumour and a spotaneous tumour, respectively (Ralph *et al*, 1977; Raschke *et al*, 1978), mouse erythroleukemia (MEL) F4-12B cell-line, derived from a spleen focus forming virus (SFFV)-induced erythroleukemia (Ostertag *et al*, 1979) and STO embryonic fibroblasts (Martin *et al*, 1975) were maintained in Special Liquid Medium (SLM) containing 10% (v/v) foetal calf serum (FCS) and 4mM glutamine, in 5% CO_2 (v/v) at 37°C. This medium is hereafter referred to as "cell growth medium". Swiss 3T3 cells were grown in Dulbecco's Modified Eagle Medium (DMEM), containing 10% (v/v) FCS, 37.5 μg/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine and 3% (w/v) sodium bicarbonate, in 5% (v/v) CO_2 at 37°C. This latter medium is hereafter referred to as "3T3 growth medium".

7.1.2 Passaging of cells.

RAW 264.7-, J774.2 and F4-12B cells adhere weakly to the surface of tissue culture flasks. To passage these cells, ~90% of the medium was removed from confluent cultures, the adherent cells were removed from the surface of the tissue culture flask using a cell-scraper, and disaggregated and dispersed in the remaining medium using an automatic pipetting aid. ~$5x10^6$ resuspended cells were seeded into 30 mls of fresh growth medium in a Nunc 175 cm^2 tissue-culture flask.

STO cells and Swiss 3T3 cells are strongly adherent to the surface of tissue culture flasks. To passage these cells, the cells were washed *in-situ* with PBS, then removed from the flask by treating with a 0.25% trypsin solution in CT (chapter **6**) buffer for 5 minutes at room temperature, after which an equal volume of the relevant cell growth medium was added in order to inhibit the trypsin. Cells were harvested by centrifugation for 5 minutes at 1200 rpm in an MSE Centaur benchtop centrifuge at room temperature, and the cells resuspended at ~$5x10^6$ cells/ml in the appropriate cell growth medium, and reseeded as above.

7.1.3 Serum starvation and -stimulation of Swiss 3T3 cells.

Swiss 3T3 cells were grown to 70% confluence in 3T3 growth medium, after which the cells were PBS washed twice *in-situ* and fresh 3T3 growth medium containing 0.3% (v/v) FCS instead of 10% (v/v) FCS was added. After 72 hours, this medium was removed by aspiration, the cells incubated in fresh 3T3 growth medium for the appropriate time and RNA extracted or nuclear extracts made.

7.1.4 Serum stimulation of RAW 264.7 cells.

RAW 264.7 cells were replated in Nunc 175 cm^2 tissue culture flasks and cultured for 72 hours, after which the medium was removed, fresh cell growth medium (ie. containing fresh 10% (v/v) FCS) was added directly to the cells for the appropriate times, then total RNA or nuclear extracts were made.

7.1.5 Culture of RAW 264.7 cells in RAW 264.7 cell conditioned medium (RAW CM).

The cell growth medium from RAW 264.7 cells, cultured for 72 hours after replating in fresh cell growth medium, was termed RAW CM, and was removed and stored at 4^oC prior to use in RAW 264.7 cell transfection experiments. Prior to use, fresh glutamine was added to a concentration of 4 mM.

7.1.6 Cell storage.

Cells were resuspended at a concentration of 10^7 cells/ml in the appropriate cell culture medium, containing 10% (v/v) glycerol and 20% (v/v) FCS, then dispensed into 1 ml "Nunc" cryotubes and stored in a well-insulated container at -70^oC for up to 4 weeks, before transfer to liquid nitrogen for long-term storage. To retrieve stored cells, cells were thawed in warm water then seeded directly into 20 mls of the appropriate cell growth medium in an 80 cm^2 tissue culture flask.

7.2 PROPAGATION OF PLASMID DNA IN BACTERIAL HOST CELLS.

7.2.1 Bacterial host cells.

Two strains of *E.coli* bacteria were used for propagation of plasmid DNA. JM83 cells (genotype *ara* δ(*lac-pro*AB) *rps*L π80*lac*ZδM15) were generally used, except when guaranteed high transformation was required, in which case library-efficient competent DH5α cells (GIBCO) (genotype F- *end*A1 *hsd*R17 (r_k^-,m_k^+) *sup*E44 *thi*-1 λ^- *rec*A1 *gyr*A96 *rel*A1 δ(*arg*F- *lac*ZYA)U169 π80d*lac*ZδM15) were used.

7.2.2 Culture of bacterial host cells.

Suspension cultures were grown in L-broth or Superbroth at 37°C, with shaking at 250 rpm in a New Brunswick G25 shaker. Colony cultures were grown on inverted 1.5% (w/v) agar/L-broth plates at 37°C. For both suspension and colony cultures, the culture medium was supplemented with ampicillin at a final concentration of 50 µg/ml, and agar/L-broth plates thus supplemented are henceforth termed "L-Amp plates".

7.2.3 Preparation of JM83 glycerol stocks.

Stationary cultures of JM83 cells in L-broth were mixed with an equal volume of a 30% (v/v) glycerol/L-broth solution, chilled on ice, then frozen at -70°C. Cells from thawed glycerol stocks were retrieved into L-broth using a sterile tip or tungsten loop.

7.2.4 Preparation of JM83 transformation-competent bacterial host cells.

For preparation of competent JM83 cells, a 20 ml overnight (o/n) suspension culture was grown, using 20 mls of L-broth and 20 µl of JM83 glycerol stock. 50 mls of L-broth was innoculated with 1 ml of the o/n JM83 culture, and grown until the absorbance of the culture at 600 nm was 0.3 units (L-broth used as zero standard). The cells were harvested by centrifugation (2500 rpm, 10 minutes, 4°C) in a Beckmann J-6B centrifuge, the supernatant discarded and the cells resuspended by gentle inversion in 25 mls of ice cold 100mM $CaCl_2$, then incubated on ice for 15 minutes. The cells were again harvested by centrifugation, as above, and resuspended in 5 mls of ice cold 100mM $CaCl_2$. The cell suspension was stored o/n at 4°C before use, and cells were maximally-competent up to 1-2 days after the described procedure.

7.2.5 Transformation of bacterial host cells with plasmid DNA.

50 µl aliquots of competent DH5α or JM83 cells were dispensed into 1.5 ml microcentrifuge tubes on ice. 1-10 ng of the chosen plasmid DNA, 5-fold diluted ligation mix (**7.3.11**) or equivalent volume of distilled water (as a negative control) was pipetted into the each tube, the contents of each tube were mixed by gentle tapping, and incubated on ice for 30 minutes. Cells were then heat-shocked for 45 seconds in a 45°C water bath, replaced on ice, and after addition of 400 µl of L-broth, were incubated at 37°C for 1 hr with shaking at 225 rpm, to allow expression of the

ampicillin-resistance gene on the plasmids transformed into the cells. Cells were then harvested by centrifugation at 13000 rpm in a microfuge for 20 seconds, and resuspended in 50 µl of fresh L-broth. All 50 µl of the cells from each tube were then spread evenly onto L-amp plates, using an ethanol/flame-sterilised glass spreader. The plates were allowed to dry for 10 minutes, then inverted and incubated at 37°C overnight to allow colonies to form.

7.3 PREPARATION, MANIPULATION AND PURIFICATION OF DNA.

7.3.1 Equilibriation of phenol and preparation of chloroform/isoamyl alcohol.

Phenol and chloroform are both used to remove protein contaminants from nucleic acid samples. Before use, phenol was equilibriated to a pH > 7.8, as unequilibriated phenol is acidic, and nucleic acids partition into the organic phase at acidic pH. For equilibriation, an equal volume of TE buffer [pH 8.0] was vigorously mixed with the phenol, the two phases allowed to separate, and the upper phase removed by aspiration. This procedure was repeated, once with TE [pH 8.0] buffer, and once with 0.1xTE buffer [pH 8.0], or until the pH of the phenolic phase, as measured using pH paper, was > 7.8. Isoamyl alcohol (IAA), a sulfactant, was added to chloroform in a 1:24 (v/v) proportion prior to use.

7.3.2 Purification and precipitation of DNA.

After crude plasmid DNA preparations and/or after enzymatic modifications of DNA, DNA was routinely purified as follows: Two volumes of a 1:1 phenol/chloroform/IAA mixture was added to the DNA sample, the sample was thoroughly mixed by vortexing, and the organic (lower) and aqueous (upper) phases were separated by centrifugation, either for two minutes at 13000 rpm using an MSE Micro Centaur, or for 10 minutes at 2500 rpm in a Beckmann J-6B centrifuge, depending on sample size; the aqueous phase was retained, and the procedure was repeated. An equal volume of chloroform/IAA was then added to the separated aqueous phase, the mixture thoroughly vortexed and centrifuged, and the aqueous phase retained, as above. Sodium acetate [pH 5.2] was added, to a final concentration of 0.2 M, and DNA precipitated with 2.5 volumes of 100% ethanol. The mixture was thoroughly vortexed, cooled for 5 minutes to 1 hour, and the DNA pelleted by centrifugation. The temperature and time of cooling, and the time of centrifugation

depend on the amount of DNA present: for recovery of small amounts of DNA, the DNA was precipitated at lower temperatures (ie. on dry ice), and the time of precipitation and the time and speed of centrifugation were increased.

7.3.3 Minipreparation of plasmid DNA.

"Minipreps." were prepared by the alkaline lysis method, essentially as described (Birnboim *et al*, 1979). Bacterial colonies were picked from L-Amp plates, and used to innoculate 5 mls of ampicillin-supplemented L-broth, which was then incubated overnight at 37°C with shaking at 250 rpm. 1ml aliquots were then dispensed into microfuge tubes, and cells harvested by microfugation at 13000 rpm for 20 seconds. Bacterial cell pellets were resuspended in 100 µl of glucose buffer (50mM glucose, 25mM Tris.Cl [pH 8.0], 10mM EDTA), then 200 µl of a freshly made solution of 1% (w/v) SDS, 0.2M sodium hydroxide (NaOH) was gently mixed with the resuspended cells, and the mixture incubated on ice for 20 minutes to allow lysis of bacteria. 150 µl of 3M NaAc was then added, the solution mixed by vigorous vortexing, incubated on ice for a further 5 minutes, then microfuged at 13000 rpm for 5 minutes to separate out the cell debris. The supernatant was further purified by a single phenol:chloroform extraction, using 400 µl of a 1:1 phenol/chloroform/IAA mixture. The resulting aqueous phase was mixed with 1 ml of 100% ethanol by vigorous vortexing and incubated at room temperature for 5 minutes to precipitate the nucleic acids, which were then pelleted by centrifugation at 13000 rpm for 10 minutes in a microfuge. The pelleted nucleic acid was dried by inversion of the tubes and pipetting away of excess liquid, then dissolved in 50 µl of TE buffer [pH 7.5].

7.3.4 Large scale preparation of plasmid DNA.

Two methods for large-scale preparation of plasmid DNA were employed, the second of which is a modification of the first and which superseded the first during the course of this project. Both methods are based on the alkaline lysis method, as described for minipreparation of plasmid DNA (Birnboim *et al*, 1979).

7.3.4.1 Method 1.

250 mls of L-broth or Superbroth, containing 50 µg/ml ampicillin, was innoculated with 1 ml of an overnight transformed bacterial cell culture and incubated for 36 hrs at 37°C, with shaking at 250 rpm, in a New Brunswick G25 shaker. Cells were harvested by cetrifugation at 3000 rpm for 15 minutes in a Sorvall GS3 rotor at

4°C. Cell pellets were incubated on ice and resuspended in glucose buffer, containing 5 mg/ml lysozyme, by gentle pipetting. After incubation of the cell suspension at room temperature for a further 5 minutes, 20 mls of a freshly-made 1% (w/v) SDS, 0.2M NaOH solution was added with gentle mixing, and the mixture incubated on ice for 20 minutes. 15 mls of 3M NaAc [pH 5.2] was then added, and the solution vigorously mixed by vortexing and shaking, then centrifuged at 6000 rpm for 15 minutes in a Sorvall GS3 rotor at 4°C, to separate out the cell debris. After removal of most of the remaining cell debris from the supernatant by filtration through gauze, nucleic acids were first precipitated, by vigorous mixing of the supernatant with 100 mls of 100% ethanol and incubation at room temperature for 10 minutes, then pelleted by centrifugation at 6000 rpm, as above. Pelleted nucleic acids were dissolved in 5 mls of 5 x TE [pH 7.5] buffer, then incubated at 37°C for 30 minutes in the presence of 40 µg/ml DNase-free RNaseA to degrade bacterial RNA. The solution was further purified by phenol/chloroform/IAA (x2) and chloroform/IAA extractions. DNA in the aqueous phase was preprecipitated, using 0.5 mls of 4 M NaCl and 3 volumes of 100% ethanol, pelleted by centrifugation at 3000 rpm for 15 minutes at 4°C in a Beckmann J-6B centrifuge, then resuspended in 2 mls of TE buffer [pH 7.5]. The plasmid DNA was purified from low MWt contaminants by gel-filtration, wherein a 1ml aliquot of the DNA solution was loaded onto a 30 cm x 1.5 cm Biogel A-50M agarose bead column equilibriated with a TE buffer [pH 7.5]/ 0.1% (w/v)/ SDS solution. 1.5 ml fractions were eluted from the loaded column using TE buffer [pH 7.5]/ 0.1% (w/v) SDS solution, and the absorbance at 260 nm and 280 nm (using TE buffer [pH 7.5]/ 0.1% (w/v) SDS solution as a blank) of each fraction was measured. The first absorbance peak at 260 nm contains the plasmid DNA, and positive fractions were pooled, precipitated by the addition of LiCl to a concentration of 0.25M and 3 volumes of 100% ethanol, pelleted, resuspended in 1 ml of distilled water, and the DNA concentration quantitated by spectrophotometry (**7.3.5**).

7.3.4.2 Method 2: using "Qiagen" kit for preparation of plasmid DNA.

250 mls of L-broth or Superbroth, containing 50 µg/ml ampicillin, was innoculated with 1 ml of an overnight trnansformed bacterial cell culture and incubated for 36 hrs at 37°C, with shaking at 250 rpm, in a New Brunswick G25 shaker. Cells were harvested by centrifugation at 3000 rpm for 15 minutes in a Sorvall

GS3 rotor at 4°C. Cell pellets were incubated on ice and completely resuspended by gentle pipetting, in 10 mls of 50 mM Tris-Cl, 10 mM EDTA [pH 8.0], 100 µg/ml RNaseA. 10 mls of a 1% SDS/ 0.2 M NaOH solution was added, and the solutions mixed by gentle inversion and incubated at room temperature for 5 minutes, after which 10 mls of chilled 3M KAc [pH 5.5] was added and the solutions mixed by gentle inversion. After 20 minutes incubation on ice, cell debris was removed from the solution by centrifugation at 6000 rpm for 15 minutes in a Sorvall GS3 rotor at 4°C and filtration of the supernatant through gauze. The filtered solution was applied to a Qiagen tip 500 column equilibriated with 10 mls of a solution containing 750 mM NaCl, 50 mM MOPS, 15% ethanol and 0.15% Triton X-100. The column was subsequently washed twice with 30 mls of a solution containing 1.0 M NaCl, 50 mM MOPS [pH 7.0] and 15% ethanol. DNA was eluted from the column with 15 mls of a solution containing 1.25 M NaCl, 50 mM Tris-Cl [pH 8.5] and 15% ethanol, precipitated at room temperature with 0.7 volumes of isopropanol, and centrifuged immediately at 15000 rpm in a Sorvall SS-34 rotor at 4°C for 30 minutes. The resulting DNA pellet was washed with 15 mls of 70% ethanol, air-dried for 5 minutes, resuspended in 1 ml of distilled water, and the DNA concentration measured by spectrophotometry (**7.3.5**).

7.3.5 Measurement of nucleic acid concentration by spectrophotometry.

The absorbance (A) at 260 nm and 280 nm of DNA and RNA solutions was measured in a 500 µl quartz cuvette, using TE, distilled water or TE/ 0.1% (w/v) SDS as a blank where appropriate. An A_{260} of 1 unit is equivalent to 50 µg/ml of plasmid or genomic DNA, and 40 µg/ml of RNA or single-stranded DNA. The OD_{260}/OD_{280} ratio gives an estimate of the purity of the nucleic acid, as pure preparations of DNA and RNA have an OD_{260}/OD_{280} ratio of 1.8 and 2.0, respectively. If the OD_{260}/OD_{280} ratio was significantly lower than the above, this was due to contaminants, which were removed by further phenol, chloroform/IAA extraction and ethanol precipitation before remeasurement of the OD_{260}/OD_{280} ratio. When the concentration of the nucleic acid in the sample was too high (A_{260} >1), the sample was diluted in distilled water and the OD_{260}/OD_{280} ratio remeasured.

7.3.6 Gel electrophoresis.

7.3.6.1 Agarose gel electrophoresis of plasmid DNA.

Plasmid DNA was routinely analysed by 1-2% (w/v) agarose gel electrophoresis. A 1% (w/v) agarose gel was made by heating 1g of agarose with 2 mls of 50 x TAE buffer and distilled water up to a volume of ~90 mls, until the agarose was fully dissolved. The solution was made up to 100 mls with distilled water, allowed to cool to ~50^{o}C, then poured into an agarose gel mould of the required size containing an appropriate well-forming comb. After allowing the agarose to set, the comb was removed and the gel placed in an electrophoresis tank containing enough 1xTAE buffer to just cover the surface of the gel. Plasmid DNA samples and the appropriate molecular size markers, to which the appropriate volume of 10x gel-loading buffer (50% (v/v) glycerol, 0.4% (w/v) bromophenol blue, in distilled water) had been added, were loaded into adjacent wells and electrophoresed at a constant voltage of ~150 volts (minigel) or ~100 volts (larger gels). Bromophenol blue migrates at ~ the same rate as linear double stranded DNA of ~300 bps in length in a 1.2% agarose gel in 1xTAE buffer. After the DNA had migrated the required distance, the agarose gel was stained in distilled water containing 1 μg/ml of ethidium bromide for up to 30 minutes, re-electrophoresed for 10 minutes, and the DNA visualized by UV-fluorescence of the ethidium bromide intercalated in the DNA on a UV trans-illumunator. A polaroid photograph of the illuminated DNA was usually taken, and the amount of DNA in each plasmid band was estimated by comparison with the known amount of DNA in molecular weight marker bands of the same UV-fluorescence.

If plasmid DNA or a particular restriction fragment was to be recovered from the agarose gel for further use, the DNA was electrophoresed as above, but on a 1.5% (w/v) low melting point agarose gel (**7.3.8**).

7.3.6.2 Polyacrylamide gel electrophoresis (PAGE).

Polyacrylamide gel electrophoresis (PAGE) of DNA and RNA was used when very accurate size resolution and/or distinction between such molecules differing in size by as little as one nucleotide was important. A stock aqueous solution of 29% (w/v) acrylamide: 1% (w/v) bis-acrylamide, henceforth termed 30% acrylamide, was stored at 4^{o}C.

For DNA sequencing and *in-vitro* DNase1 footprinting, 6% (w/v) denaturing polyacrylamide gels were used. A solution containing 20 mls of 30% (w/v)

acrylamide, 45g of urea, 10 mls of 1xTBE buffer and distilled water to ~90 mls was dissolved with gentle heating, and the volume then adjusted to 100 mls with distilled water. 400 µl of a 10% (w/v) ammonium persulphate (APS) solution and 120 µl of N, N, N', N' Tetramethylethylenediamine (TMED) were added by stirring, and the solution immediately poured into a siliconized glass 0.4 mm thick sequencing gel mould, and a well-forming comb inserted. The gel was allowed to polymerize for 1 hour, then pre-electrophorezed for 20 minutes in 1x TBE buffer in a vertical gel tank at a constant current of 40 mA. Samples, denatured at 90°C for 2 minutes, were then loaded and electrophoresed at 40 mA constant current for 1-3 hours.

For analysis of single stranded RNA molecules protected in RNase protection experiments, denaturing polyacrylamide gel solution was made as above, except that the final concentration of acrylamide was 5% (w/v). The gel was 0.75 mm thick, and was pre-electrophoresed for 20 minutes and electrophoresed, upon loading of denatured samples, at ~250 volts (constant voltage), for the required time.

For purification of 5' end-labelled double-stranded oligonucleotide probes (**7.4.4**) and EMSA analysis of nucleoprotein binding to DNA (**7.10.1**), 0.75 mm thick 5% non-denaturing polyacrylamide gels were used, the former run at 100 volts, constant voltage, in 1xTBE buffer, and the latter in 0.25xTBE buffer at 150 volts, constant voltage.

For analysis of UV-crosslinked DNA-nucleoprotein adducts in UV-crosslinking experiments (**7.10.2**), gels were made as follows: A 7.5% SDS-PAGE solution was made by mixing 5 mls of 30% acrylamide stock solution with 5 mls of a 1.5 M Tris-Cl [pH 8.8] / 0.4% SDS stock solution and 10 mls of distilled water. 30 µl of 10% APS and 10 µl of TMED were added, and the solution poured into a 0.75 mm thick gel mould, ovelayed with butan-2-ol and allowed to polymerise for 1 hour. A 1% acrylamide stacking gel solution was meanwhile prepared by mixing 1.5 mls of a 30% acrylamide stock solution with 2.5 mls of a 0.5M Tris-Cl [pH 6.8]/ 0.4% SDS stock solution and 6 mls of distilled water. 30 µl of 10% APS and 10 µl of TMED were added, and after removal of the butan-2-ol from the polymerized SDS-PAGE gel, the stacking gel solution was poured onto the top of the SDS-PAGE gel, ovelayed with butan-2-ol, and allowed to polymerize for 1 hour. The gel, containing samples,

was electrophoresed at 20-25 mA (constant current) in an electrolyte containing 0.192 M glycine, 0.025 M Tris and 0.1% SDS.

7.3.7 Restriction enzyme digestion of plasmid DNA.

Restriction enzyme digestion of plasmid DNA was carried out in the smallest volume possible, within the limits defined by the volume of the DNA sample and the volume of restriction endonuclease(s) used in the digestion, the latter not exceeding 10% of the final volume in the digest, due to the high glycerol concentration of restriction enzyme stock solutions. The buffer used in restriction enzyme digests was supplied with each restriction enzyme by the manufacturers, usually as a 10x or 5x stock solution. Digests were made up to the required volume using distilled water, then incubated for 1 hour at the temperature recommended by the suppliers (usually 37^{o}C). For double digests, if both restriction enzymes worked optimally in different buffers, either a compromise buffer was used (for example "1 Phorall" buffer), in which both enzymes worked, or first one enzyme was used, then the constituency and volume of the digestion buffer altered so that the second enzyme would work, and a further incubation of 1 hour was performed. Generally, between 1-5 units of restriction enzyme were used per µg of DNA. For digestion of miniprep. plasmid DNA samples, in which bacterial RNA is present, 10 µl of plasmid DNA was digested with the appropriate restriction enzymes and digestion buffers in a total volume of 50 µl, in the presence of 50 µg/ml of DNase1-free RNaseA. Restriction enzyme-digested DNA was analysed by agarose gel electrophoresis and ethidium bromide staining (**7.3.6.1**).

7.3.8 Purification of DNA fragments from agarose gels.

DNA restriction fragments were resolved by 1.5% (w/v) low melting point (LMP) agarose gel electrophoresis, visualized by ethidium bromide staining and UV illumination or by exposure to autoradiographic film if radioactive, excised from the gel using a clean scalpel, and placed into 10 mls of low salt buffer (LSB) at 65^{o}C for 1 hour, with vortexing every 15 minutes, to melt the agarose. The solution was then incubated for a further ~10 minutes at 45^{o}C, during which time an Elutip-D-column was equilibriated, first with 5 mls of high salt buffer (HSB), then with 5 mls of LSB at 45^{o}C, in each case by passing the buffer through the column at high pressure using a syringe. 5 mls of the DNA/agarose solution was then applied to the column in a

similar manner, after which the column was washed with 10 mls of LSB at 45°C, before eluting the DNA from the column with 0.6 mls of HSB. The remaining 5 mls of the DNA/agarose solution was applied to a second Elutip-D-column in an identical manner. The resulting 1.2 mls of DNA in HSB was precipitated with 3 volumes of 100% ethanol, pelleted by microcentrifugation, washed with 70% ethanol, vacuum-dried and resuspended in an appropriate volume of distilled water. The concentration of the resuspended DNA fragment was estimated by 1.2% (w/v) agarose minigel electrophoresis and ethidium bromide staining, in comparison to a known amount of the appropriate co-electrophoresed molecular weight markers.

7.3.9 Generation of DNA fragments by the polymerase chain reaction (PCR) using plasmid DNA as template.

7.3.9.1 Design of PCR oligonucleotides.

In each case, two single-stranded oligonucleotide (oligo) primers, synthesised and purified as described in **7.3.13** and generally ~20-40 bases in length, were designed such that they were complementary to opposite strands and opposite ends of the DNA sequence of interest. Oligonucleotides contained a "GCGCGC" sequence at their 5' ends, to protect them from exonuclease degradation, followed by a restriction enzyme recognition sequence 5' to the sequence complementary to the template, allowing subcloning of the PCR-generated DNA fragment into the appropriate restriction sites of the chosen plasmid vector. The 3' end of oligos was either a G or a C base if possible, in order to enhance priming from the oligonucleotide.

7.3.9.2 PCR reactions.

The template for PCR reactions was linearized plasmid DNA, containing the sequence of interest, diluted to a concentration of 10^{-11}g/ µl. 10^{-11}g of plasmid template DNA was combined, in a PCR reaction tube, with 25 pmols. of each PCR primer, 10 µl of Taq polymerase buffer (provided with Taq enzyme by the manufacturers), 2 µl from 5 mM stock solutions (in distilled water) of each of dATP, dTTP, dGTP and dCTP, 6 µl of 25mM $MgCl_2$, 0.5 µl of Taq polymerase and distilled water to a final volume of 100 µl. The solution was overlayed with 50 µl of PCR oil, then incubated in a PCR machine (Perkins-Elmer) for 25-30 of the following cycles: 1) 95°C for 1 minute; 2) 55°C for 1 minute; 3) 72°C for 1 minute. Reactions were incubated for 10 minutes at 72°C after the final cycle, then gradually cooled to 4°C.

Each reaction was extracted with one volume of chloroform, ethanol precipitated, analysed by 1.5% (w/v) LMP agarose gel electrophoresis (**7.3.6.1**) and the relevant band purified (**7.3.8**), restriction digested (**7.3.7**) and resuspended in distilled water.

7.3.9.3 PCR-mediated site directed mutagenesis of plasmid DNA sequences.

Four PCR oligonucleotide primers (1, 2, 3 and 4) were used in these reactions. Primers 1 and 4 were complementary to ~20 bases of opposite DNA strands, at the opposite termini of the DNA region of interest, and were designed with the same features as the PCR primers used in (**7.3.9.1**). Primers 2 and 3 contained sequences complementary to opposite strands of the DNA site to be mutated, and primed in opposite directions, such that primers 1 and 2 used in one PCR reaction, and primers 3 and 4 used in another PCR reaction, would generate overlapping double stranded DNA fragments, containing point mutations in the overlapping region, spanning the DNA region of interest. Primers 2 and 3 were both designed in effectively three sections: the central 10 base section contained the point mutations of interest; the 3' section, beginning at the 3'-most point mutation, contained 15 bases perfectly complementary to the relevant region of the template DNA; the 5' section of primer 2 contained 10 bases of sequence perfectly complementary to the 3' section of primer 3, and vice-versa, so that primers 2 and 3 contained 30 bases of overlapping complementary sequence. Primers 1 and 2 were used together- and primers 3 and 4 together in PCR reactions under the same conditions as in (**7.3.9.2**), using linearized plasmid DNA containing the DNA sequence of interest as template. The resulting overlapping DNA fragments were purified and resuspended in distilled water, as above (**7.3.9.2**). Primers 1 and 4 were then used in a third PCR reaction under the same conditions as (**7.3.9.2**), except that plasmid template DNA was omitted, and ~10 ng of each of the overlapping DNA fragments generated above was includled in the reaction, resulting in the generation of a single DNA fragment of the required length, containing the required point mutations, and containing the required restriction enzyme recognition sites at its termini, allowing ligation of the DNA fragment into a plasmid vector.

7.3.10 Preparation of DNA for ligation.

7.3.10.1 Dephosphorylation of plasmid 5' termini using calf intestinal alkaline phosphatase (CIP).

Plasmid DNA was digested with the appropriate restriction enzymes, generally in a total volume of 30 µl, then the exposed 5' termini of the DNA dephosphorylated using calf intestinal alkaline phosphatase (CIP), as follows. The digest volume was made up to 200 µl with 17 µl of CIP buffer, 0.1 units of CIP per ~2.5 µg of plasmid, and distilled water, and incubated at 37°C for 30 minutes, after which a further 0.1 units of CIP was added and the incubation repeated. For blunt-ended restriction sites, the incubation was for 15 minutes at 37°C and 15 minutes at 56°C, again repeated. 160 µl of distilled water and 40 µl of 10x STE (100mM Tris-Cl [pH 8.0], 1M NaCl, 10mM EDTA) was then added, to stop the reaction, and the mixture extracted twice with phenol/chloroform/IAA and then twice with chloroform/IAA. The DNA was precipitated twice with 100% ethanol, then resuspended at a concentration of ~100 ng/ µl in distilled water.

7.3.10.2 Blunt-ending of DNA fragments and/or linearized plasmid.

When the sequence of the DNA region of the plasmid vector of choice did not contain the required restriction enzyme recognition sites into which a particular DNA restriction fragment could be directly ligated, the DNA fragment was first blunt-ended before ligation into an appropriate blunt-ended restriction site in the plasmid vector. If no naturally blunt-ended restriction site within the required region of the plasmid vector was available, the plasmid was first restriction-digested within the required region, then blunt-ended before ligation with the blunt-ended DNA fragment.

For blunt-ending of ~0.5-1 µg of DNA, the DNA was combined with 2.5 µl of 10x Klenow buffer (supplied with the enzyme by the manufacturer), 1 unit of Klenow enzyme, 1 µl of 2mM dNTPs (2mM of each of dATP, dCTP, dTTP and dGTP) and distilled water to a total volume of 25 µl. The solution was incubated for 30 minutes at room temperature, stopped by the addition of 1 µl of 0.5M EDTA, then phenol/chloroform/IAA extracted (x2), chloroform/IAA extracted, ethanol precipitated and resuspended in distilled water at a concentration of 10-100 ng/ µl.

7.3.11 Ligation of DNA fragments into plasmids.

If, after isolation of DNA fragments, it was necessary to restriction enzyme digest those fragments prior to their use in ligations, phenol/chloroform/IAA extraction and ethanol precipitation of plasmids and fragments was employed prior to the ligations. Ligation reactions were in the smallest possible volume, typically 20 µl.

50 ng of linearized plasmid vector DNA was combined in separate microfuge tubes with a range of estimated amounts of restriction fragment DNA (1:1 to 10:1 (mol/mol) fragment:plasmid), plus 4 µl of 5x ligation buffer (supplied by the manufacturers with the T4 DNA ligase), 1 unit of T4 DNA ligase and distilled water to a final volume of 20 µl. Linearized plasmid in the absence of DNA fragment, and in the presence or absence of DNA ligase, was used as a control. Reactions were incubated for 4 to 15 hours in a water bath at 14°C, then diluted 5-fold with distilled water and stored at -20°C until required for transformation of bacterial cells (**7.2.5**). Because ligation buffer contains polyethylene glycol (PEG), which favours inter- rather than intra- molecular ligation, plasmid DNA was religated (after cutting out a segment of DNA from the plasmid, for example) using 1 µl, instead of 4 µl, of ligation buffer in the 20 µl ligation reaction.

7.3.12 Sequencing of plasmid DNA.

DNA subcloned into plasmids pUC18 or p0GH was sequenced using the dideoxy-mediated chain termination method devised by Sanger (Sambrook *et al*, 1989). Primers used are listed in Table **6.4**. All such sequencing reactions were performed using the protocol and Sequenase Version 2.0 kit supplied by USB, [α-^{35}S] (1000 Ci/ mmol)-dATP as the radioactive label. ~4 µg of DNA was used per reaction, either from purified large-scale plasmid preparations, or from Qiagen column-purified minipreparations of DNA (**7.3.4.1** and **7.3.4.2**). Prior to sequencing, 20 µg of double-stranded plasmid template DNA was denatured for 5 minutes at room temperature with 0.2 M NaOH, after which ammonium acetate was added to a final concentration of 1.5 M. The DNA was precipitated using 3 volumes of 100% ethanol, pelleted, washed with 70% ethanol, vacuum dried and dissolved at a concentration of 1 µg/ml in distilled water.

The G and A nucleotides of ^{32}P-end-labelled DNA fragments used in *in-vitro* DNase1 footprinting reactions (**7.9**) were sequenced using the chemical degradation sequencing method of Maxam and Gilbert (Sambrook *et al*, 1989), using a kit as recommended by the suppliers (NEN). 2 µl of [γ-^{32}P] end-labelled DNA was mixed with 18 µl of distilled water and 2 µl of piperidine formate, the mixture was incubated for 15 minutes at 37°C, frozen in dry ice, freeze-dried, dissolved in 30 µl distilled water, frozen in dry ice and freeze-dried. The resulting DNA pellet was dissolved in

100 µl of aqueous 1 M piperidine, incubated at 90°C for 30 minutes, frozen on dry ice then freeze-dried. The DNA pellet was then subjected to 3 cycles of the following: dissolving in 30 µl distilled water, frozen, freeze-dried. Finally, the DNA pellet was dissolved in 50 µl of footprint gel-loading buffer (95% (v/v) formamide, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue), and stored at -20°C until required.

7.3.13 Synthesis of oligonucleotides.

Oligonucleotides were synthesized at the Beatson Institute on an Applied Biosystems model 381A DNA synthesizer according to the manufacturers instructions. 5' trityl groups were removed by the machine, and the DNA immobilized on a column. The DNA was eluted from the column in 29% (v/v) ammonia, by passing the ammonia continuously through the column for three 5-minute periods separated by 20 minute intervals. The resulting DNA-ammonia solution was sealed in a glass vial, incubated overnight at 55°C, then made 0.3 M with respect to ammonium acetate, and precipitated with 3 volumes of ethanol on dry ice for for 20 minutes. DNA was pelleted by centrifugation at 10000 rpm in a Sorvall SS-34 rotor for 15 minutes, redissolved in 3 mls of aqueous 0.2 M NaAc, precipitated with 3 volumes of 100% ethanol and 0.2M NaAc, then pelleted by centrifugation, as above. The DNA pellet was washed with 70% (v/v) ethanol, freeze-dried and dissolved in 1 ml of distilled water. After quantitation by spectrophotometry (**7.3.5**), oligonucleotides were stored at -20°C until required.

7.3.14 Preparation of double-stranded oligonucleotides.

5 µg of each of two complementary single-stranded oligonucletides were combined with 10 µl of 10x TE buffer, 10 µl of 1 M NaCl and distilled water to a final volume of 100 µl. The solution was placed in a boiling water bath for 1 minute, and then allowed to cool gradually to room temperature in the water bath, after which the sample was removed and stored at -20°C until required.

7.4 PREPARATION OF RADIOACTIVE DNA AND RNA PROBES.

7.4.1 Random-primed radiolabelling of DNA probes.

All DNA probes used for hybridization to Northern and Southern blots were labelled for 30 minutes at 37°C with [α-^{32}P]-dCTP, using a random-priming kit as recommended by the suppliers (Boehringer-Mannheim). After the labelling reaction,

the labelled probe was separated from unincorporated nucleotides on a Nick-column (Pharmacia) equilibriated with 0.1x SSC, 0.1% (w/v) SDS. Radiolabelled probes were denatured by boiling for 2 minutes prior to use.

7.4.2 Uniform labelling of anti-sense RNA riboprobes.

Riboprobes were used for hybridization to Northern- and Southern blots, and for hybridization in solution in RNase protection assays to total RNA prepared from cell-lines. pBluescript SK^-- (pSK^-) or pSPT19 vector containing the DNA fragment of interest was linearized using the appropriate restriction enzyme, to prevent the use of vector sequences as template by T3 or T7 RNA polymerase. Vector thus linearized was phenol/chloroform/IAA extracted, ethanol precipitated and dissolved in distilled water at a concentration of 1 μg/μl. 1μg of linearized vector was then used with the SP6/T7 transcription kit (Boehringer-Mannheim) and with T3- or T7 RNA polymerase supplied by GIBCO-BRL, as recommended by the suppliers, to generate [α-^{32}P]-UTP-labelled anti-sense RNA transcripts of the desired probe sequence. Probe, with a specific activity of ~10^8cpm/μg, was purified using a Nick-column (Pharmacia) equilibriated with 0.1x SSC, 0.1% (w/v) SDS, and was denatured by boiling for 2 minutes prior to use.

7.4.3 5' end-labelling of DNA fragments for *in-vitro* DNase1 footprinting.

50 μg of plasmid containing the DNA sequence of interest was restriction enzyme digested at the end to be labelled, and the digestion was checked for completion using agarose minigel electrophoresis (**7.3.6.1**). The 5' termini of fully-digested plasmid DNA were dephosphorylated using calf intestinal alkaline phosphatase, as described (**7.3.10.1**), and redissolved in TE to give a 1 μg/μl stock solution. 10 μg of this DNA stock was combined with 5 μl of 1 Phor-all buffer (Pharmacia), 5 mM DTT, 2 mM spermidine, 100 μCi of [γ-^{32}P]-ATP (3000 Ci/mmol), 7 units (1 μl) of T4 polynucleotide kinase (Northumbria Biologicals) and distilled water to a final volume of 50 μl, and incubated for 30 minutes at 37°C. The reaction was stopped by incubation for 10 minutes at 70°C, the solution was phenol/chloroform/IAA- and chloroform/IAA-extracted and ethanol precipitated (**7.3.2**), then resuspended in TE containing 0.1 M KAc and reprecipitated with 3 volumes of 100% ethanol. The DNA pellet was redissolved in distilled water, and digested with the appropriate second restriction enzyme to release the 5' end-labelled

DNA fragment of interest from the plasmid vector.. The 5' end-labelled probe and plasmid vector thus generated were resolved by 1.5% (w/v) LMP gel electrophoresis (**7.3.6.1**), visualized by autoradiography, and purified (**7.3.8**). The specific activity of the probe was estimated by resuspending a 1 µl aliquot of probe DNA in 5 mls of scintillation fluid and measuring β counts using a scintillation counter, and was of the order of 10^6 cpm/µg.

7.4.4 5' end-labelling of double-stranded oligonucleotides.

All double stranded oligonucleotides (oligos) synthesized were blunt-ended. 5' end labelling was achieved by combining 200 ng of double-stranded oligo with 1 µl of 1-Phor-all buffer, 2mM spermidine, 20 µCi of [γ-^{32}P]-ATP (3000 Ci/µl), 7 units (1 µl) of T4 polynucleotide kinase and distilled water up to a final volume of 10 µl, and incubating this solution for 30 minutes at 37^{o}C. After addition of 1 µl of 10x gel loading buffer (50% (v/v) glycerol, 0.4% (w/v) bromophenol blue), reactions were loaded directly onto an 8% (w/v) polyacrylamide/ 1xTBE buffer gel (**7.3.6.2**) and electrophoresed for 2 hours at 100 V (constant voltage). The 5' end-labelled double-stranded oligo probe was visualized by autoradiography (Kodak XAR film) at room temperature for 3-5 minutes, excised from the gel using a clean scalpel, and eluted overnight in 1 ml of TE [pH 8.0] buffer at 37^{o}C.

7.5 DETECTION OF SPECIFIC MAMMALIAN GENOMIC DNA SEQUENCES BY SOUTHERN BLOTTING.

7.5.1 Isolation of mammalian genomic DNA.

Cells were harvested as described (**7.1.2**). Cell pellets were resuspended in 1 ml of TE [pH 8.0], (5x10^7 cells/ml), and 10 mls of extraction buffer (10mM Tris.Cl [pH 8.0], 0.1M EDTA, 0.5% (w/v) SDS, 20 µg/ml DNase-free RNaseA). The cell suspension was incubated with gentle shaking for 1 hr at 37^{o}C, then proteinase K added, to a final concentration of 0.1 mg/ml, and the solution incubated for 3 hrs at 50 ^{o}C, with periodic gentle shaking. The solution was then cooled to room temperature, and extracted twice with 1 volume of phenol. DNA was precipitated with 3 volumes of ethanol and 0.2 M NaCl, collected by spooling onto a bent pasteur pipette, air-dried for 15 minutes, then dissolved at 4^{o}C over a period of three days, in 1 ml of TE [pH 8.0] buffer per 2x10^7 cells in the original cell pellet. The DNA solution was then

dialysed in TE [pH 8.0] buffer overnight at 4°C, to remove any remaining salt and/or ethanol, then stored at 4°C until required. The DNA was quantitated by spectrophotometry immediately prior to use (**7.3.5**).

7.5.2 Southern blotting of DNA.

20 µg of DNA was restriction enzyme digested (**7.3.7**) overnight, using 50 units of restriction enzyme, then for a further 6 hours after the addition of a further 20 units of restriction enzyme. Agarose gel loading buffer was added, and the DNA electrophoresed overnight at 40 mA on a 1% (w/v) agarose gel (**7.3.6.1**). The gel was then soaked for 30 minutes in 500 mls of a solution containing 0.5 M NaOH and 1.5 M NaCl (to denature the DNA) then twice for 30 minutes in a solution containing 3 M NaCl and 0.5M Tris-Cl [pH 7.5] (to neutralize the gel), both with constant gentle agitation. After neutralization, the gel was transferred in an inverted orientation onto 3MM paper on a solid support above a reservoir (~ 1 litre) of 20x SSC, into which the edges of the 3MM paper were submerged. A piece of nitrocellulose was cut to the same size as the gel, wet in distilled water, soaked for 10 minutes in 20x SSC, then placed on top of the gel. Clingfilm was arranged around the perimeter of the gel in such a way as to ensure that 20x SSC from the reservoir would only diffuse through the gel. Three pieces of 3 MM paper cut to the size of the gel were placed on top of the nitrocellulose, and paper towels and a weight placed on top of the 3MM paper. The arrangement was left overnight, to allow the capillary transfer (by the 20xSSC) of the DNA out of the gel and onto the nitrocellulose paper, after which the nitrocellulose was baked for 1 hour in a vacuum oven at 80°C, to fix the DNA onto the filter. The filter was then pre-wetted in 5xSSPE, incubated for 5 hours at 42°C in 50 mls of pre-hybridization solution, then hybridized overnight in the same solution, to which the chosen riboprobe or DNA probe (**7.4.1** and **7.4.2**) had been added. Prior to addition to the hybridization mixture, riboprobes were heated at 90°C for 2 minutes, and DNA probes were boiled for 2 minutes then chilled for 1 minute on ice. Hybridized membranes were then sequentially washed (each time in ~200 mls) as follows: in 2x SSC, 0.1% SDS at room temperature for 30 minutes; in 2x SSC, 0.1% SDS at 65°C for 30 minutes; in 0.1x SSC, 0.1% SDS at 65°C for 30 minutes, after which excess moisture was removed from the mebranes by gentle blotting with 3MM

paper, before sealing in thin polythene bags and exposing to Kodak-X-OMAT AR film at -70°C.

7.6 DETECTION OF SPECIFIC RNA SEQUENCES.

7.6.1 Isolation of total RNA from mammalian cells.

Total cellular RNA was prepared using the RNAzol B method, according to the manufacturer's instructions. Briefly, cell growth medium was removed from ~$5x10^7$ cells in a Nunc 175 cm^2 flask, the cells were washed once *in-situ* with PBS, lysed with 10 mls of RNazol B, transferred to a Falcon 2059 (polypropylene) tube and homogenized by pipetting. The homogenate was mixed thoroughly with 1 ml of chloroform, incubated on ice for 15 minutes, then centrifuged at 10000 rpm for 15 minutes, after which the upper phase was removed and total RNA precipitated with 1 volume of ispopropanol for 15 minutes at -20°C. The pellet was washed with 70% (v/v) ethanol, vacuum dried, then resuspended (~$5x10^7$ cells/ 100 µl) in distilled water pre-treated with diethylpyrocarbonate (DEPC, an irreversible inhibitor of RNases) (DEPC-treated water was prepared by incubating a 0.1% (v/v) DEPC-water solution overnight, then autoclaving, which destroys the DEPC). RNA was quantitated by spectrophotometry (**7.3.5**), diluted with an equal volume of 100% ethanol, then stored at -20°C.

7.6.2 Detection of specific sequences in total RNA by Northern blotting.

20 µg of total cellular RNA was freeze-dried, and resuspended in 15 µl of RNA sample loading buffer (1x MOPS buffer, 2.2M formaldehyde, 50% (v/v) formamide, 5% (v/v) glycerol, 0.4% (w/v) bromophenol blue, 50 µg/ml ethidium bromide). The RNA was heated at 90°C for 2 minutes, briefly placed on ice, then resolved by denaturing agarose gel electrophoresis (also see **7.3.6.1**); gels contained 1.5% (w/v) agarose, 1x MOPS buffer, 2.2 M formaldehyde, and distilled water to a final volume of 250 mls, and were electrophoresed in 1x MOPS buffer for 2 hours at 100 volts (constant voltage). The gel was photographed to check for RNA integrity and equal loading between samples, transferred to a solid support and blotted overnight (**7.5.2**) in 10x SSC, onto a nylon genescreen membrane that had been pre-wetted in distilled water and soaked in 10x SSC for 15 minutes prior to use. The RNA was fixed to the membrane by baking for 1 hour at 80°C in a vacuum oven, and

prehybridized, hybridized and washed essentially as described in **7.5.2**, except that prehybridization and hybridization of Northern blots with riboprobes was at 65°C. Blots were analyzed for RNA loading by first stripping of probe from hybridized membranes by boiling of membranes for 10 minutes in distilled water containing 0.1% (w/v) SDS, then pre-hybridizing and hybridizing the membrane with a random-primed (**7.4.1**) rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) double-stranded DNA probe at 42°C.

7.6.3 Detection of specific sequences in total RNA by RNase protection analysis.

20 µg of total cellular RNA or yeast control RNA and ~10^5 cpm of the relevant [α-^{32}]-UTP-labelled antisense RNA riboprobe (**7.4.2**) in 0.3 M NaAc, was ethanol precipitated with 3 volumes of ethanol, pelleted by microcentrifugation at 4°C and dissolved in RNA hybridization buffer (solution A in the Ambion RNase protection assay (RPA) kit supplied by AMS Biotechnology Ltd.) in the following manner: Samples were sequentially vortexed, briefly microfuged, incubated at 95°C for 3 minutes, vortexed, then briefly microfuged. Resuspended samples were incubated for a further 2 minutes at 95°C, then incubated in a waterbath at 45°C for 24 hours, such that sample tubes were kept almost entirely submerged in the water for the duration of the incubation. All total cellular RNA samples and one yeast RNA sample were then digested with an RNaseA/RNaseT1 mixture (typically 5 µg/ml and 100 units/ml, respectively) in 200 µl of buffer Bx, supplied with the RPA kit, while a second sample containing yeast RNA was mock-digested in 200 µl of solution Bx. 300 µl of solution Dx (RPA kit) was then added to each sample and RNA samples pelleted, then resuspended in 10 µl of RPA kit gel-loading buffer. Protected RNA fragments were resolved by denaturing PAGE (**7.3.6.2**) and autoradiography. Size markers were pUC18 plasmid Sau3AI restriction enzyme fragments, 5' end-labelled with [γ-32P]-ATP and polynucleotide kinase essentially as described in (**7.4.4**), then resuspended in RPA gel loading buffer. Immediately prior to gel-loading, size-markers and samples were incubated at 95°C for 3 minutes.

7.7 PREPARATION OF CRUDE NUCLEAR EXTRACTS FROM MAMMALIAN CELLS.

Unless otherwise stated, all manipulations were conducted at 4°C, and all solutions used contained the following, which are either a reducing agent (dithiothreotol, DTT), or protease-, serine/threonine phosphatase (β-glycerophosphate)- or tyrosine phosphatase (sodium orthovanadate)- inhibitors: 1 mM DTT, 500 μM phenylmethylsulphonyl fluoride (PMSF), 500 μM benzamidine, 10 mM sodium butyrate, 10 mM β-glycerophosphate, 2 mM levamisole, 50 μM sodium orthovanadate and 1 μg/ml of each of leupeptin, aprotinin, bestatin and pepstatin A. All inhibitors were added from concentrated stock solutions (see chapter **6**) immediately prior to use. A 1 mM sodium orthovanadate stock solution was diluted with distilled water from a 100 mM stock solution, adjusted to pH 8.0 (turns banana yellow), boiled until colourless then cooled to 4°C immediately prior to use.

7.7.1 Large-scale preparation of nuclear protein extracts.

Nuclear proteins were prepared essentially as described by Plumb *et al*, 1986). Approximately 10^{10} cells were harvested, and sequentially washed with 200 mls of each of the following solutions, after each of which the cells were pelleted at 2000 rpm at 4°C for 20 minutes in an IEC centrifuge: a) phosphate-buffered saline (PBS); b) TMS buffer (0.25 M sucrose, 5 mM $MgCl_2$, 100 mM Tris-Cl [pH 7.5]); c) TMS buffer containing 0.25% (v/v) Triton X-100; d) TMS buffer. The cell nuclei were then resuspended in 100 mls of TMS buffer. DNA content was estimated by taking two 100 μl aliquots, and adding 900 μl of distilled water, then 9 mls of 1M NaOH to each. The aliquots were then sonicated, and the average nucleic acid concentration measured by spectrophotometry (**7.3.5**). Nuclei were then pelleted and resuspended in TMS buffer, at a nucleic acid concentration of ~5-10 mg/ml. The volume of the sample was measured, NaCl added dropwise with stirring to a final concentration of 0.4 M, and the non-histone proteins extracted from the nuclei by gentle stirring on ice for 10 minutes. Samples were then clarified, first by centrifugation at 17000 rpm in a Sorval SS-34 rotor, then for 1 hour at 55000 rpm in a T-1270 rotor in a Beckman benchtop ultracentrifuge. The volume of the supernatant was meausured, and solid ammonium sulphate added to 0.35 g/ml and dissolved by stirring on ice for 20 minutes. The precipitated crude nuclear protein was pelleted by centrifugation at 17000 rpm in a Sorval SS-34 rotor, and the pellet resuspended in ~5 mls of E_{50} buffer (50 mM ammonium sulphate, 20 mM Hepes [pH 7.9], 5 mM $MgCl_2$, 0.1 mM EDTA,

0.1% (v/v) Brij-35, 20% glycerol in distilled water). The solution was dialyzed overnight against 1 litre of storage buffer (SB), clarified by centrifugation in a Beckman benchtop ultracentrifuge at 55000 rpm, then stored in aliquots at -70°C prior to use.

7.7.2 Small-scale preparation of nuclear extracts.

Nuclear extracts were prepared using a modification of a protocol used by Wyke *et al* (Beatson Institute). $5x10^7$ cells were washed twice *in-situ* with PBS at 4°C, scraped, resuspended in PBS, transferred to Universal tubes, and pelleted for 5 minutes at 2000 rpm in a Beckman J-6B centrifuge. Cell pellets were resuspended by pipetting, in 10 mls of hypotonic buffer (10 mM Tris-Cl [pH 7.5], 10 mM NaCl, 1.6 mM $MgCl_2$, 1 mM $CaCl_2$, in distilled water) in which 0.25% (v/v) Triton X-100 had been dispersed by stirring, and incubated on ice for 5 minutes to allow cell lysis. Nuclei were pelleted in a Beckman J-6B centrifuge at 2000 rpm for 10 minutes, the supernatant discarded, pellets resuspended by pippetting, in 10 mls of TMS buffer, the centrifuged as above at 2500 rpm for 10 minutes. The supernatant was aspirated, nuclei resuspended in ~200 µl of storage buffer, and the solution transferred to Beckmann ultracentrifuge T-1270 rotor tubes. The volume of the suspension was measured, NaCl added dropwise, to a final concentration of 0.4 M, and mixed in by gently flipping the tubes. Tubes were incubated on ice for 15 minutes, with periodic flipping, then centrifuged for 1 hour in a Beckman benchtop ultracentrifuge at 55000 rpm. Nuclear extracts, thus clarified, were stored in 20 µl aliquots at -70°C.

7.7.3 Determination of protein concentration in crude nuclear extracts.

Approximate protein concentration of nuclear extracts was determined using Coomassie Protein Assay Reagent, as recommended by the suppliers. The Coomassie reagent was diluted 1:1 with distilled water, and 1 ml added to 5 µl of nuclear extract (using 5 µl of storage buffer alone as a control) or to bovine serum albumin samples of known protein concentration. The OD_{595} of these samples was measured by spectrophotometry, and the values obtained from the bovine serum albumin standards used to plot a standard curve of OD_{595} vs. protein concentration (in µg/ml), from which the approximate protein concentration of nuclear extract samples was determined.

7.8 PREPARATION OF MAMMALIAN CELL NUCLEI FOR NUCLEAR RUN-ON ANALYSIS.

Nuclei were prepared by a modification of the method of Bentley *et al* (ICRF, London: personal communication). Nuclei were prepared as in (**7.7.2**), except for the following: Inhibitors (**7.7**) were omitted from the solutions, and cells were gently resuspended in RSB (10 mM Tris [pH 7.5], 10 mM potassium glutamate, 1.5 mM $MgCl_2$) containing 0.25% Triton X-100, dispersed by stirring. After 5 minutes incubation on ice, cells were lysed by vigorous shaking for 1 minute, the caps of the Universals loosened, and nuclei pelleted as in (**7.7.2**). Nuclei were not washed, but were gently resuspended in 500 µl of glutamate buffer (40% (v/v) glycerol, 125 mM potassium glutamate, 10 mM Hepes [pH 8.0], 5 mM $MgCl_2$, 2 mM DTT, 1 mM EGTA), transferred to microfuge tubes, then pelleted for 1 minute by microfugation at 3000 rpm, and all but ~40 µl of the supernatant removed. Nuclear pellets were resuspended by brief vortexing and, if required, gentle pipetting, then stored at -70°C until required.

7.9 *IN-VITRO* DNase1 FOOTPRINTING ANALYSIS OF NUCLEOPROTEIN BINDING TO DNA.

The footprinting protocol used was essentially as described by Plumb *et al* (1986). Unless otherwise stated, experiments were performed on ice, and storage buffer and nuclear protein extracts contained DTT and inhibitors, at the concentrations described in **7.7**. Between 0-80 µl of crude nuclear protein extract (**7.7.1**), was incubated in a solution containing 30 µg/ml of poly (dI-dC)-(dI-dC), 1 µl (~10000 cpm) of 5' end-labelled footprinting probe (**7.4.3**) and storage buffer to a final volume of 100 µl, ice for 1 hour. A 2 mg/ml DNase1 stock solution in distilled water was diluted in storage buffer containing 1 mM $CaCl_2$, to give DNase1 solutions at concentrations of (**A**) 200 µg/ml and (**B**) 20 µg/ml. After the 1 hour incubation, between 1-10 µl of DNase1 solution **A** was added to all except control tubes, to which between 1-10 µl of DNase1 solution **B** was added. Tubes were immediately vortexed and incubated at room temperature for 30 seconds, after which 100 µl of STOP buffer (1.5 mls = 1.3 mls of 10x TE [pH 8.0] buffer, 70 µl 10% (w/v) SDS, 60 µl of proteinase K (from a 10 mg/ml stock), 15 µl of tRNA (from a 10 mg/ml stock) and 38

µl of 4 M NaCl) was added to each tube. Samples were then incubated for 30 minutes at 37°C, 90°C for 2 minutes, sequentially phenol/chloroform/IAA- then chloroform-extracted. 5 M LiCl was then added to a final concentration of 0.1 M, the DNA was precipitated with 3 volumes of 100% ethanol, pelleted, freeze-dried and resuspended in 10 ml of footprinting gel-loading buffer (95% formamide (v/v) formamide, 0.1% (w/v) xylene cyanol FF, 0.1% (w/v) bromophenol blue). Samples, plus the resuspended G+A sequencing reaction of the 5' end-labelled footprinting probe (**7.3.12**), were denatured for 3 minutes at 90°C, snap-cooled on dry ice, thawed and a 5 µl aliquot immediately loaded on a pre-electrophoresed footprinting polyacrylamide gel and electrophoresed for 1-3 hours (**7.3.6.2**).

7.10.1 ELECTROPHORETIC MOBILLITY SHIFT ASSAY (EMSA).

Depending on the DNA binding characteristics of the particular transcription factor being analysed, various parameters, such as temperature and time of incubation, presence or absence sodium orthovanadate and/or β-glycerophosphate, REDOX conditions sodium chloride concentration and GTP concentration, were altered between assays (see Results section). However, unless othersise stated, storage buffer used in EMSAs contained DTT and protease and phosphatase inhibitors, such that the final concentration of these reagents in EMSAs was eqivalent to that in storage buffer used to prepare nuclear extracts (**7.7**).

Between 0.5 µg and 2 µg of nuclear extract, as prepared in **7.7.2**, was incubated with 3 µg of poly(dI-dC)-(dI-dC) and 0-200 ng of the appropriate unlabelled double-stranded competitor oligonucleotide (**7.3.14**) in a final volume of 20 µl of storage buffer, and the mixture incubated on ice for 30 minutes, after which 1 ng of the appropriate 5' end-labelled double-stranded oligonucleotide probe (**7.4.4**) was added. If GTP was to be included in any tubes, it was also added at this point, and all tubes (- or + GTP) were incubated for a further 20 minutes at room temperature, whereas if no GTP was to be added to any of the tubes, all tubes were incubated for a further 60 minutes on ice. Samples were then loaded onto a pre-electrophoresed- non-denaturing 5% polyacrylamide gel (**7.3.6.2**), electrophoresed for ~ 2 hours, and gels vacuum dried and exposed to Kodak X-OMAT-AR film at -70°C.

7.10.2 UV-CROSSLINKING OF TRANSCRIPTION FACTORS TO DNA.

Complimentary single-stranded oligonucleotides were synthesized (**7.3.13**) with bromodeoxyuridine (BrdU) substituted for thymidine at chosen positions (within proposed transcription factor binding site(s), and annealed as described (**7.3.14**), to produce double-stranded BrdU-substituted oligonucleotides, which were 5' ^{32}P end-labelled (**7.4.4**) and used in EMSAs (**7.10.1**). The wet EMSA gel was then exposed overnight at 4°C to Kodak-XAR5 fast film, allowing visualization of retarded complexes. Retarded complexes were excised from the gel and UV-irradiated (320 nm) for 20 minutes, then placed into SDS sample buffer (62.5 mM Tris-Cl [pH 6.8], 5% SDS, 5% β-mercaptoethanol, 10% glycerol and 2% bromophenol blue) for 1 hour. Gel slices were then placed directly into the wells of a 7.5% SDS-PAGE gel (**7.3.6.2**) and resolved by electrophoresis and autoradiography. ^{14}C-labelled methylated protein molecular weight markers (Amersham) were used as size markers.

7.11 NUCLEAR RUN-ON ANALYSIS OF GENE TRANSCRIPTIONAL ACTIVITY.

7.11.1 Nuclear run-on reaction.

Nuclear run-on analysis was carried out essentially according to a protocol used by Bentley *et al* (ICRF, London: personal communication). 40 µl of cell nuclei, prepared as described in **7.8**, were thawed, mixed gently on ice with 160 µCi (8 µl) of [^{32}P-α]-UTP, 0.5 µl of 1M creatine phosphate (SIGMA) in 10 mM Hepes [pH 8.0], 1.2 µl of a 2 mg/ml stock of creatine kinase (SIGMA) in glutamate buffer and 1 µl of a nucleotide triphosphate solution (50 mM ATP, 25 mM GTP, 25 mM CTP), then incubated at 37°C for 15 minutes. Total RNA was immediately prepared from the cells using 800 µl of RNAzol B and 400 µl of chloroform (**7.6.1**). RNA pellets were washed with 70% ethanol, freeze-dried, and dissolved in a final volume of 90 µl of a solution containing 0.5% SDS (w/v) and 1 mM EDTA, by vortexing and heating at 95°C for 2 minutes. 10 µl of 3M NaOH was added to each sample, samples were incubated on ice for 10 minutes to degrade the RNA to ~100 base fragments, then neutralized by the addition of 100 µl of 0.48M Hepes.

7.11.2 Measurement of TCA-precipitatable material in nuclear run-on RNA.

TCA precipitatable material in each RNA sample was measured as follows: A 2 µl aliquot from each neutralized sample was combined with 83 µl of distilled water, 10 µl of 100% (w/v) and 5 µl of tRNA (10 mg/ml), the mixture was incubated on ice for 1 hour, then mixed with 900 µl of 10% (w/v) TCA. Samples were then pipetted onto prewet cellulose filters (Whatman GF-C), and filtered through a Millipore harvester. The filters were washed once with 10 mls of ice-cold 10% (w/v) TCA and once with 10 mls of 100% ethanol, dried under a lamp for 1 hour, vortexed in 5 mls of scintillation fluid, then β scintillation counted in an LKB 1215 Rackbeta scintillation counter. Equivalent counts of TCA-precipitatable material (~1-2x 10^7 cpm/ml) were added directly to small Universal tubes containing pre-hybridized nitrocellulose filters upon which the chosen plasmid DNA containing the probe sequence of interest was immobilized.

7.11.3 Immobilization of plasmid DNA on nitrocellulose filters.

Plasmids, containing the probe DNA sequences of interest, were immobilized on nitrocellulose filters using a slot-blotting procedure. Nitrocellulose filters were pre-wet in 2x SSC buffer, then placed on the slot-blotter. 2 µg of plasmid DNA was diluted in 50 µl of 5x SSC buffer, denatured by incubation at 95°C for 3 minutes, rapidly cooled on ice, then immediately pipetted onto the slot-blotting apparatus, with the vacuum applied until all of the sample in each well had disappeared. The DNA was fixed onto the nitrocellulose by incubation at 80°C in a vacuum oven for 1 hour.

7.11.4 Hybridization of [^{32}P-α]-UTP labelled hydrolized nuclear RNA to plasmid probe sequences.

Nitrocellulose filters were prehybridized for ~4 hours at 65°C in a solution containing 6x SSC, 10 mM EDTA, 5x Denhardt's solution, 0.5% (w/v) SDS and 100 µg/ml of sonicated salmon sperm DNA. Without allowing filters to dry, strips ~1 cm wide and containing one "slot" each of all of the immobilized plasmids, were cut from the filter, and placed into prehybridization buffer at 65°C in small Universal containers, in such a volume (~1.6 mls) that the nitrocellulose strip was just covered by buffer. The required volume of [^{32}P-α]-UTP-labelled RNA (~10^7 cpm) (see **7.11.2**) was added to each Universal, and the filters hybridized at 65°C with shaking, for ~72 hours. After hybridization, filters were rinsed in 1x SSC buffer to remove the SDS, sealed in a bag with 10 mg/ml RNaseA in a solution containing 0.3M NaCl, 10

mM Tris-Cl [pH 7.5] and 5 mM EDTA at 37°C for 30 minutes, then sequentially washed, as described for Northern- and Southern blot filters (see **7.5.2** and **7.6.2**). After washing, excess moisture was removed from filters using 3MM paper, and filters were exposed to Kodak X-OMAT AR film at -70°C.

7.12 *IN-VIVO* DNase1 HYPERSENSITIVITY OF CHROMATIN.

Cell nuclei were prepared on a large scale, as described in **7.7.1**, except that the only inhibitor used was PMSF, 0.1 mM EGTA was present in all solutions, and nuclei were finally resuspended at a nucleic acid concentration of ~1 mg/ml in RSB (**7.8**) containing 0.1 mM $CaCl_2$. A 2 mg/ml stock of DNase1 in distilled water was diluted, using RSB containing 0.1 mM $CaCl_2$, to give 200 µg/ml and 20 µg/ml stocks of DNase1. Aliquots from these stocks, or of RSB containing 0.1 mM $CaCl_2$ (control), were gently mixed with 1 ml samples of nuclei on ice, at final concentrations of 0 µg/ml (control), 0.1 µg/ml, 0.2 µg/ml, 0.5 µg/ml, 0.7 µg/ml, 1 µg/ml, 5 µg/ml and 10 µg/ml of DNase1. Samples were incubated at 37°C for 5 minutes, reactions stopped by addition of 9 mls of STOP solution (10 mM Tris-Cl [pH 7.5], 5 mM EDTA, 0.5% (w/v) SDS, 40 µg/ml proteinase K), incubated overnight at room temperature, with gentle agitation. The solution was then extracted sequentially, twice with 1 volume of phenol, once with 2 volumes of phenol/chloroform/IAA, and once with 1 volume of chloroform/IAA. Sodium chloride was added to a concentration of 0.2 M, nucleic acids ethanol precipitated overnight at -20°C, pelleted, dissolved in 2 mls of 5x TE [pH 8.0] buffer, and digested overnight at 4°C with 100 µg/ml of DNase-free RNaseA. Samples were then sequentially extracted with phenol/chloroform/IAA and chloroform/IAA, 0.2 M NaCl was added, the nucleic acids ethanol precipitated overnight, pelleted and resupended in TE buffer, as described in **7.5.1**. DNA was restriction enzyme digested, electrophoresed, Southern blotted and probed as described in **7.5.2**.

7.13 TRANSFECTION OF PLASMID DNA INTO RAW 264.7-, STO- AND MEL CELLS.

All transfectants used the human growth hormone (hGH) gene as a reporter gene. All cell incubations and all cell growth media (unless otherwise stated) were as

described in **7.1.1**. In all transient transfections, a positive control plasmid pHSVTKGH, in which a Herpes Simplex Virus (HSV) promoter drives hGH reporter gene expression, and a negative control plasmid p0GH, containing a promoterless hGH gene, were used in transfection (co-transfected with the chosen reference plasmid) of each of the transfected cell types. In a particular cell type, corrected transient transfection expression values for reporter plasmids and for the pTKGH positive control were obtained by first deducting background counts (usually ~300 cpm measured by the γ-counter), then measuring the reference plasmid gene activity in each transfectant, and adjusting the measured hGH activity for each transfectant accordingly. The adjusted hGH activity of the the zero control plasmid (p0GH) was then deducted from the adjusted hGH values obtained for the other transfectants. Reporter gene activity was compared between cell types by asssuming the HSV TK promoter is equally active in all cell types, and arbitrarily assigning the corrected hGH activity produced by the pHSVTKGH plasmid transfectants as 1 unit in all cell types. Reporter plasmid hGH activity in each cell type was then expressed as a multiple of the hGH activity of the pHSVTK plasmid.

7.13.1 Calcium phosphate-DNA co-precipitation.

7.13.1.1 Transient transfection of F4-12B MEL- and RAW 264.7 cells.

Conditions used in transient transfections of F4-12B MEL cells were those previously shown to be optimal for transient transfection of these cells by this method (McLeod *et al*, 1991). For transient transfection of RAW 264.7 cells, transfections were essentially the same as those for F4-12B cells, except that total transfected DNA, amount of reporter- and reference plasmids, pH of 2x HBS, and time of incubation of the cells with the calcium phosphate-DNA precipitate were varied, in order to optimise transfections. Because I was unable to detect expression of either the pHSV β-gal- or the luciferase (pSV40-LUC) reference plasmids in RAW 264.7 cells (see Results and Discussion sections), only the method for transient transfection of F4-12B cells is outlined here. Briefly, 10^6 cells were plated on 9 cm petri dishes in cell growth medium and grown overnight (**7.1.1**). A calcium phosphate-DNA precipitate was made by adding, with gentle mixing, 100 µl of sterile 2.5 M $CaCl_2$, 10 µg of pHSV β-gal or pSV40-LUC, 20 µg of hGH gene-containing reporter plasmid, 10 µg of carrier DNA (pUC18 plasmid) and sterile TE buffer [pH 8.0], to a total

volume of 1 ml, in the order: TE, $CaCl_2$, carrier DNA, reference plasmid, reporter plasmid. This solution was added to 1 ml of sterile 2x Hepes buffered saline (HBS) [pH 7.05] (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4.H_2O$, 12 mM dextrose, 50 mM Hepes), and the solution mixed twice by gentle pipetting, and incubated at room temperature for 30 minutes to allow a precipitate to form. 1 ml of each precipitate was added to duplicate F4-12B petri-dishes (set up as above), and the plates incubated overnight, after which the medium was aspirated from the cells, fresh cell growth medium added, and the cells incubated for a further 20 hours before taking medium samples (for hGH assay: **7.14.5**) and harvesting the cells (for β-galactosidase (**7.14.2**) or luciferase assay (**7.14.3**)).

7.13.1.2 Harvesting of the cells and cell lysis.

Cells were harvested as follows: All cell growth medium was aspirated from the plates, the cells were scraped in 1 ml of PBS, transferred to microfugfe tubes, microfuged for 30 seconds at 2500 rpm, the supernatant aspirated, and the cell pellets stored at -20°C until required. Pellets were resuspended in 200 µl of 0.25 M Tris-Cl [pH 7.8] by vortexing, then lysed by 3 cycles of: 5 minutes on dry ice, 5 minutes at 37°C. Debris from lysed cells was pelleted by microcentrifugation for 2 minutes at 13000 rpm, and the supernatant retained at -20°C until required.

7.13.1.3 Stable transfection of RAW 264.7 cells.

RAW 264.7 cells were transfected using the calcium phosphate-DNA co-precipitation technique (see **7.13.1.1**), except that 10 µg of reporter plasmid DNA, 10 µg of carrier DNA, and 1 µg of Homer 6 plasmid, which contains a gene conferring resistance to the antibiotic geneticin (G418), were co-precipitated. Control cells were transfected either with only 10 µg of reporter plasmid DNA and 10 µg of carrier DNA, or were mock-transfected. After aspiration of precipitate and addition of new growth medium to the cells (see **7.13.1.1**), cells were incubated for 48 hours in growth medium, split 1 in 5 and replated in fresh cell growth medium (**7.1.1**), then selected with 900 µg/ml of G418 for 2 weeks (more if all of the control cells had not died in this time). Once selected, >100 G418 resistant colonies were pooled, replated in fresh cell growth medium containing 900 µg/ml of G418 to expand the cell numbers, then stored in aliquots in liquid nitrogen (**7.1.6**) until required.

7.13.2 Lipofectin-mediated transfection.

7.13.2.1 Transient transfection of F4-12B MEL- , STO- and RAW 264.7 cells.

Conditions for transfection of these cell types by the lipofection method were optimised by variation of the amount of Lipofectin reagent (GIBCO BRL) used, the amount of reporter- and reference plasmids used, and the time of transfection (ie. the time of incubation of the cells with the Lipofectin-DNA mixture). Because Lipofectin reagent tends to adhere to pipette tips, each Lipofectin sample was pipetted using a new pipette tip, and pipetting of samples containing Lipofectin reagent was kept to a minimum. Optimised transfection protocols are as follows:

7.13.2.1.1 RAW 264.7 cells.

Cells were harvested from an ~80% confluent Nunc 175 cm^2 tissue-culture flask containing ~2x 10^7 cells, and used to inoculate ~200 mls of fresh cell growth medium (**7.1.1**), such that the final concentration of cells was ~2x 10^5 / ml. 2 ml aliquots of cells were plated onto Falcon 3001 35 mm tissue-culture plates and incubated overnight, after which the medium was aspirated, cells PBS-washed *in-situ*, then incubated (**7.1.1**), generally for < 1 hour, in 1 ml of OPTIMEM medium (GIBCO BRL), containing 4 mM glutamine, until required for transfections. Transfection reactions were mixed in a 96-well tissue culture dish. In one well, 50 µl of distilled water was mixed with 50 µl of Lipofectin reagent (GIBCO BRL), by pipetting twice. In a separate well, 3 µg of reporter plasmid was mixed with 0.3 µg of pSV2ASPAP (encoding secreted alkaline phosphatase (SPAP) protein) reference plasmid in a total volume of 100 µl of distilled water. This DNA mixture was then mixed, by pipetting 4 times, with the Lipofectin/water mixture, and the solution incubated at room temperature for 15-45 minutes. Plates containing cells in OPTIMEM medium were removed from the incubator, and 20 µl of the Lipofectin-DNA mixture pipetted onto each plate. Plates were briefly swirled to disperse the transfection mix, and returned to the incubator for ~15 hours, after which the OPTIMEM medium was removed by aspiration, 1 ml of fresh cell growth medium was added and the cells incubated for a further 24 hours; 100 µl samples of medium were then removed and assayed for the presence of SPAP (**7.14.4**), and the plates returned to the incubator. After a further incubation of 24 hours, cells were effectively in RAW CM (**7.1.5**), and were washed twice with PBS prior to incubation in the following media for 20 hours: 1 ml of RAW CM (**7.1.5**), in the presence or absence of 2 µg/ml bacterial cell wall

lipopolysaccharide (LPS) (supplied by DIFCO, resuspended at 2 mg/ml in distilled water, and stored in aliquots, initially at -20°C, and at 4°C once thawed); 1 ml of fresh cell growth medium containing fresh 10% FCS (**7.1.1**), in the presence or absence of 2 μg/ml of LPS. After this incubation, promoter activity was measured by measuring hGH activity in samples of media taken from the cells.

7.13.2.1.2 F4-12B MEL- and STO cells.

Harvesting, plating and transfection of F4-12B- and STO cells were carried out as described for RAW 264.7 cells, except for the following differences: ~$5x10^5$ cells were plated in 4 mls of cell growth medium, onto Falcon 3004 60 mm tissue culture plates, incubated overnight, PBS washed *in-situ*, then incubated in 2 mls of OPTIMEM medium, containing 4 mM glutamine, until required for transfections (ie. for ~1 hour). For STO cell transfections, Lipofectin-DNA mixtures were as for RAW 264.7 cells, while for F4-12B transfections, the Lipofectin/distilled water mixture consisted of 70 μl of water and 30 μl of Lipofectin. For both STO- and F4-12B cells, 65 μl of the Lipofectin-DNA mixture was added to each plate, and plates were then incubated for 5 hours before removal of the OPTIMEM and addition of 2 mls of cell growth medium. Plates were incubated for a further 24 hours, 100 μl of medium was removed for the SPAP assay (**7.14.4**), then plates were incubated for a further 48 hours and medium was assayed for hGH activity (**7.14.5**).

7.13.2.2 Stable transfection of RAW 264.7 cells.

~5x 10^6 cells at ~80% confluence in a Nunc 80 cm^2 tissue culture flask were PBS-washed twice *in-situ*, then incubated in 5 mls of OPTIMEM medium containing 4 mM glutamine prior to transfection. 3 μg of reporter plasmid and 0.3 μg of the Homer 6 plasmid, containing the neomycin resistance gene, were combined with a Lipofectin/distilled water mixture, as described in **7.13.2.1.1**. The whole of the 200 μl Lipofectin-DNA mixture was added to one flask, and the cells incubated in OPTIMEM for 15 hours, as described in **7.13.2.1.1**, after which the OPTIMEM was removed and 10 mls of fresh cell growth medium was added to each flask. Stably-transfected cells were selected and stored as described in **7.13.1.3**.

7.14 ASSAYS FOR PROTEIN PRODUCTS OF GENES CONTAINED IN TRANSFECTED PLASMIDS.

7.14.1 Assay for protein concentration in cell lysates.

In assays for protein products of transfected genes in cell lysates, it was important to measure the concentration of total protein in cell lysates so that different cell lysates could be directly compared. This was carried out using 5 µl aliquots of cell lysates, essentially as described in **7.7.3.**

7.14.2 β-galactosidase assay.

This assay (Hall *et al*, 1983) involves the hydrolysis of o-nitrophenyl-β-D-galactopyranoside (ONPG) by β-galactosidase produced within cells transfected with the bacterial β-galactosidase gene, resulting in the appearnace of a yellow colour when cell lysates from transfected cells are incubated with the ONPG substrate at 37°C. Briefly, 0.5 mls of Solution **1** (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol) was added to 30-60 µl of cell lysate in a plastic cuvette, 100 µl of Solution **2** (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 2 mg/ml ONPG) was added, with vigorous mixing, the solution was incubated at 37°C for 30-90 minutes, the reaction stopped by vigorous mixing with 250 µl of 1M Na_2CO_3, and the absorbance of the solution at 420 nm determined spectrophotometrically, using the mock-transfected cell lysate reaction as a blank control. Typically, 50 µl of STO- or MEL transfected cell lysate resulted in an A_{420} reading of between ~0.2 and 1.0 after incubation for 1 hour at 37°C.

7.14.3 Luciferase assay.

In this assay (deWet *et al*, 1987), firefly luciferase protein present in transfected cell lysates is incubated with its substrate, luciferin protein, in the presence of ATP, resulting in a fluorescence, directly proportional to the levels of luciferase in the cell-lysate, and measurable in a Luminometer. Briefly, cells transfected on 9 cm petri dishes (see **13.1.1**) were PBS washed, then lysed *in-situ* in 1 ml of lysis buffer (25 mM Hepes [pH 7.8], 15 mM $MgSO_4$, 4 mM EGTA, 1% (v/v) Triton X-100). Cell debris was removed by microcentrifugation of samples at 13000 rpm at 4°C for 3 minutes. A 1 mM stock of luciferin was made by dilution in luciferase assay buffer (25 mM Hepes [pH 7.8], 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, 2 mM ATP), and 30 µl of this stock was mixed with 300 µl of luciferase assay buffer and 20-100 µl of cell-lysate, and the luciferase present assayed by fluorescence in a Luminometer, using the mock-transfected cell lysate reaction as a blank control.

7.14.4 Assay for secreted alkaline phosphatase (SPAP).

100 µl of cell growth medium was removed from transfected cells (see **13.2**) for use in the SPAP assay (Wallace *et al*, personal communication; Henthorn *et al*, 1988). Cell growth medium contains high levels of phosphatase activity, which is sensitive to heat-treatment; in contrast, SPAP is highly resistant to heat-treatment (Wallace *et al*, personal communication; Henthorn *et al*, 1988). Hence, cell growth medium from transfected cells was incubated at 65°C for 2 hours prior to use, then briefly microfuged, and 50 µl aliquots transferred to plastic cuvettes. DEA buffer (1M diethanolamine, 0.28M NaCl, 0.5 mM $MgCl_2$, pH 9.85), stored at 4°C, and a 100 mM stock of p-nitrophenylphosphate (PNPP) in DEA buffer, stored at -20°C, were used, immediately prior to the assay, to make a 5 mM stock of PNPP in DEA buffer, and 1 ml of this solution was pipetted onto each heat-treated sample. Phosphatase treatment of PNPP produces p-nitrophenol, which has a yellow colour; thus reactions were incubated at 37°C until yellow colour was seen to develop, and SPAP activity in samples was determined spectrophotometrically, by measuring absorbance at 405 nm of heat-treated medium from transfected cells, using heat-treated medium from mock-transfected cells as a blank control. Typically, SPAP assay of 50µl of cell growth medium from transiently-transfected RAW 264.7 cells resulted in an A_{405} reading of between 0.2 and 1.0 after 45 minutes at 37°C. Prior to assays using medium from transfected cells, a stock of cell growth medium was heat-treated as above, and used to make dilutions of non-heat-treated cell growth medium, whose absorbances at 405 nm were plotted on a graph, versus % (v/v) of non-heat-treated medium in the sample, in order to determine the linear range of the SPAP assay, and A_{405} measurements of between 0.2 and 1.0 were within this linear range.

7.14.5 Assay for human growth hormone (hGH).

This assay is based on the work of Selden *et al* (1986) and uses the Allegro dual growth hormone-specific monoclonal antibody system as recommended by the supplier (Biogenesis Ltd.). Briefly, hGH secreted by transfected cells into the cell growth medium is recognized by two anti-hGH protein murine monoclonal antibodies, to different hGH protein epitopes, one of which is [^{125}-I]-labelled and the other of which is biotinylated and linked to avidin-coated glass beads. hGH present in the cell growth medium becomes sandwiched between the two antibodies, thus

allowing linkage of [^{125}-I] with the glass beads in amounts proportional to the levels of hGH present in the sample of cell growth medium. The level of [^{125}-I] thus linked to the glass beads is monitored by its γ-particle emission, using a Beckmann -γ-counter. 200 µl samples of medium were removed from transiently transfected cells at the indicated times (see **13.2.1.1** and **13.2.1.2**), and between 5 µl and 100µl of each sample was assayed for human growth hormome (hGH) activity. Typically, 10 µl of cell growth medium from MIP1α promoter pM-350 transiently transfected unstimulated RAW 264.7 cells produced ~10^4 γ-counts per minute, compared to a background count of ~300 cpm.

PART 3: RESULTS.

CHAPTER 8: LOCALIZATION AND SEQUENCING OF THE MIP1α GENE IN A LAMBDA EMBL3 POSITIVE GENOMIC CLONE, λ141.

8.1 OVERVIEW.

In order to study the transcriptional regulation of a gene, it is necessary to know- and to have access to- the sequence of the gene and its flanking (potential regulatory) sequences, in order that they can be analysed by various techniques that will be described in the following chapters. Prior to this study, only the cDNA sequence of the murine MIP1α gene was known (Davetalis *et al*, 1988). Hence, it was necessary to identify and characterize a genomic clone containing the MIP1α gene and required flanking sequences.

The λEMBL3 vector is a replacement viral vector used for cloning large (up to 20 kbp) fragments of genomic DNA in *E.Coli*. This vector contains cloning sites consisting of Sal1, BamH1 and EcoR1 restriction enzyme recognition sites; hence, a genomic DNA partial Sau3AI partial digest can be inserted into the BamH1 sites. Prior to this study, a λEMBL3 murine (DBA/2J) adult liver genomic library (supplied by Clonetech Laboratories, USA) had been screened in this laboratory using a MIP1α cDNA antisense RNA riboprobe generated from the pSK-MIP-cDNA plasmid (Table **6.1**), and one positive clone, λ141, had been identified. The following section describes how I characterized λ141, by a combination of restriction mapping, Southern blotting, sub-cloning and sequencing, confirming the presence of the MIP1α gene and ~5 kbps of 5-' and ~1 kbp of 3' flanking sequence within the λ clone.

8.2 RESTRICTION MAPPING OF λ141.

Prior to this study, Southern blot analysis of restriction enzyme-digested J774.2 macrophage cell-line DNA, probed with [^{32}P-α]-UTP-labelled MIP1α cDNA antisense RNA riboprobe generated from the pSK-MIP-cDNA plasmid (Table **6.1**), demonstrated that the MIP1α gene is contained within an ~8.4 kbp EcoR1 fragment,

an ~3.3 kbp EcoR1/HindIII fragment, an ~7 kbp HindIII fragment (that overlaps the described EcoR1 fragment), and an ~26 kbp BamH1 fragment, and a crude restriction map of the genomic DNA surrounding the MIP1α gene in J774.2 cells was deduced (M.Plumb, personal communication) (figure **8.2a**).

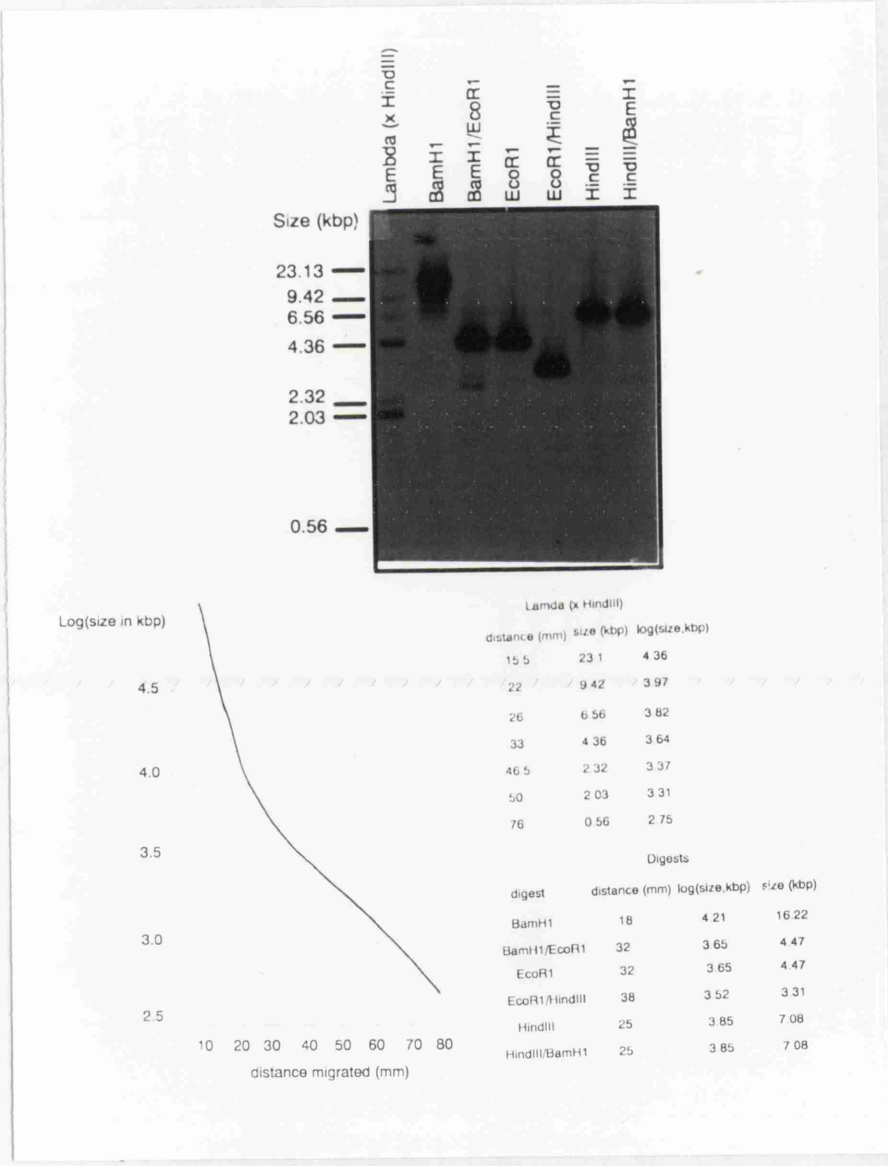
I therefore carried out a similar examination of clone λ141, knowing that, if the MIP1α gene were contained within λ141, its digestion with the same panel of restriction enzymes as that used in the analysis of the J774.2 genomic DNA should result in the production of at least some MIP1α gene-containing restriction fragments of the same size as those obtained in the J774.2 genomic DNA analysis. Hence, the λ141 DNA was restriction enzyme digested, the digests were resolved by agarose gel electrophoresis, Southern blotted, then probed with [^{32}P-α]-labelled MIP1α cDNA riboprobe (figure **8.1**), allowing a restriction map of the MIP1α gene-containing locus in the λ141 clone to be deduced (figure **8.2b**).

As shown in figure **8.2**, the restriction maps obtained for the MIP1α locus by J774.2 genomic DNA- and λ141 DNA analyses are similar, though because the λEMBL genomic library contains a partial Sau3AI genomic digestion, it is clear that the λ141 BamH1 sites are artefactual (figures **8.2a** and **8.2b**). Hence, the only unexpected difference between J774.2 and λ141 restriction maps is the different location of one of the EcoR1 sites (figure **8.2**). This difference is due to a rearrangement in the DNA during the generation of the genomic library by Clonetech Laboratories (M.Plumb and D.Cook, personal communication), so that λ141 sequences to the right of the HindIII site situated to the right of the MIP1α gene in figure **8.2b** are not contiguous with the MIP1α gene locus sequences contained in the rest of λ141. Nevertheless, the presence of three out of four equivalent restriction sites in λ141 and J774.2 genomic DNA suggested that at least ~70% of λ141 is a legitimate MIP1α gene-containing genomic clone.

Further restriction mapping of clone λ141 was therefore carried out, and as shown in figures **8.1** and **8.2b**, an ~4.4 kbp EcoR1 restriction fragment of clone λ141 appears to contain the entire MIP1α gene. Hence, this restriction fragment was cloned into the EcoR1 site of the pUC18 plasmid (p18E7, Table **6.1**) and more extensively

Figure 8.1: Genomic mapping of MIP1α gene coding sequences in Lambda EMBL3 genomic clone λ141 by Southern blot analysis.

DNA from Lambda EMBL3 genomic clone λ141 was purified and 20 μg aliquots digested with the restriction endonucleases shown above the autoradiograph. A Southern blot of these digested DNA samples was probed with a MIP1α [α- ^{32}P]-UTP-labelled antisense riboprobe synthesized from linearized pSK-MIPcDNA plasmid template. The position of λ(xHindIII) molecular weight DNA markers electrophoresed adjacent to the DNA samples are shown to the left of the autoradiograph. Below the autoradiograph, measured distances migrated by electrophoresed molecular weight markers and genomic DNA fragments is shown. From these measurements, the size (in kbps) of genomic restriction fragments containing the MIP1α gene coding sequences were calculated.



Lamda (x HindIII)

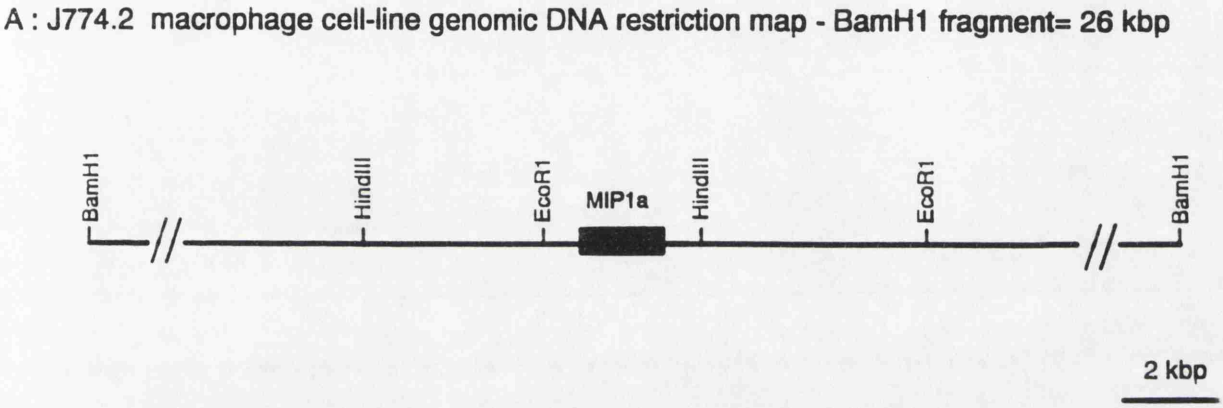
distance (mm)	size (kbp)	log(size,kbp)
15.5	23.1	4.36
22	9.42	3.97
26	6.56	3.82
33	4.36	3.64
46.5	2.32	3.37
50	2.03	3.31
76	0.56	2.75

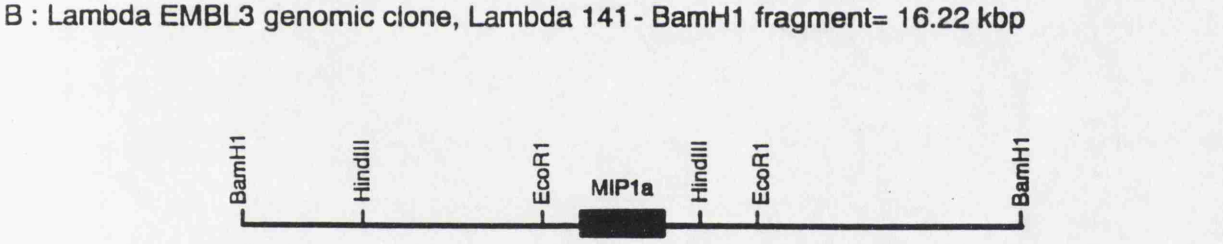
Digests

digest	distance (mm)	log(size,kbp)	size (kbp)
BamH1	18	4.21	16.22
BamH1/EcoR1	32	3.65	4.47
EcoR1	32	3.65	4.47
EcoR1/HindIII	38	3.52	3.31
HindIII	25	3.85	7.08
HindIII/BamH1	25	3.85	7.08

Figure 8.2: Restriction maps of the MIP1α gene locus for the enzymes used in Southern blot analysis.

Restriction maps of the MIP1α gene locus: **A**) in J774.2 cells; **B**) in Lambda clone λ141. MIP1α gene coding sequences are denoted by a shaded box, and the scale of the diagram (in kbps) and the restriction enzymes used in the Southern blot analysis are as shown.





characterized. As for the Southern blotting experiment (described above), this mapping involved the digestion of the clone with a panel of restriction enzymes, alone and in combination. Digestion of plasmid p18E7 with Sst1 produced two Sst1 restriction fragments, which were subcloned into pUC18 (clones p18SL3 and p18S2: see Table **6.1**), and restriction mapped. Figures **8.3** and **8.4** show such analyses of clones p18E7 and p18SL3, while figure **8.5** shows such an analysis of clone p18S2.

The data from figures **8.3-8.5** allows a restriction map of clone p18E7 to be drawn, as shown in figure **8.6**. A number of Acc1 restriction enzyme sites are not marked on figure **8.6**, as this enzyme cuts so frequently within p18E7, that the exact number and location of several of these sites cannot be accurately shown.

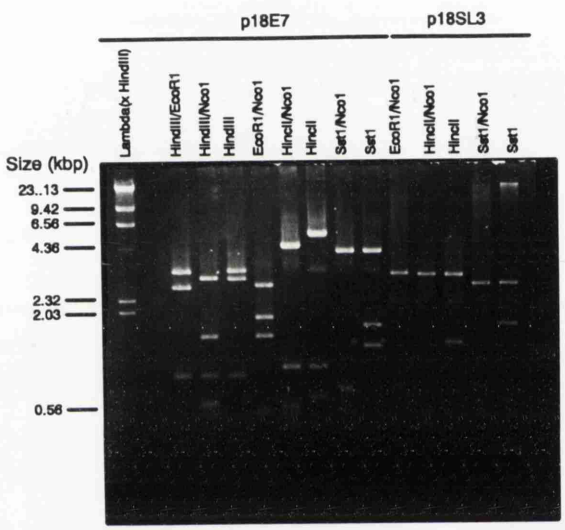
8.3 SEQUENCING OF PLASMID CLONES DERIVED FROM λ141.

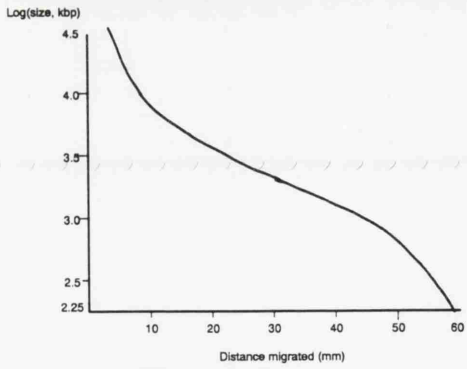
The MIP1α gene coding sequences are contained in an ~3.3 kbp EcoR1/ HindIII restriction fragment (figures **8.1** and **8.6**). By comparison with the published MIP1α cDNA sequence (Davetalis *et al*, 1988), mapped restriction sites (figure **8.6**) were identified that are present in the MIP1α coding sequence. Thus, there are single HincII- and Nco1 restriction sites present in the MIP1α cDNA, and the ~3.3 kbp λ141 EcoR1/HindIII restriction fragment contains two HincII sites and three Nco1 sites (figure **8.6**), one of each of which must be located within the MIP1α gene coding region. Hence, subcloning of the appropriate restriction fragments into pUC18 plasmid vector and sequencing the DNA in both directions from these HincII and Nco1 sites would confirm the presence of the MIP1α gene.

Sequencing of DNA subcloned into the multiple cloning site (mcs) of plasmid pUC18 was by the di-deoxy chain termination method devised by Sanger (Sambrook *et al*, 1989), using the Sequenase Version 2.0 sequencing kit. Two oligonucleotide primers, M13 Universal and M13 Reverse (Table **6.3**), which prime from sequences on either side of the pUC18 mcs, are supplied with this kit, and were used to sequence the p18E7-derived Nco1 restriction fragment N4 (figure **8.6** and Table **6.1**), confirming the presence of the MIP1α gene in λ141.

Figure 8.3: Restriction enzyme mapping of MIP1α gene-containing genomic-subclone plasmid p18E7 and its derivative p18SL3.

5 µg aliquots of p18E7 or p18SL3 plasmid DNA were digested with a panel of restriction enzymes, alone or in combination, as shown above the photograph. The digested DNA was electrophoresed on a 1.2% (w/v) agarose gel and stained with ethidium bromide. Molecular weight markers (λxHindIII) electrophoresed adjacent to the plasmid DNA samples are shown. Distance migrated by the molecular weight markers and plasmid restriction fragments was measured, and the size (in bps) of each restriction fragment calculated, as shown below the photograph.



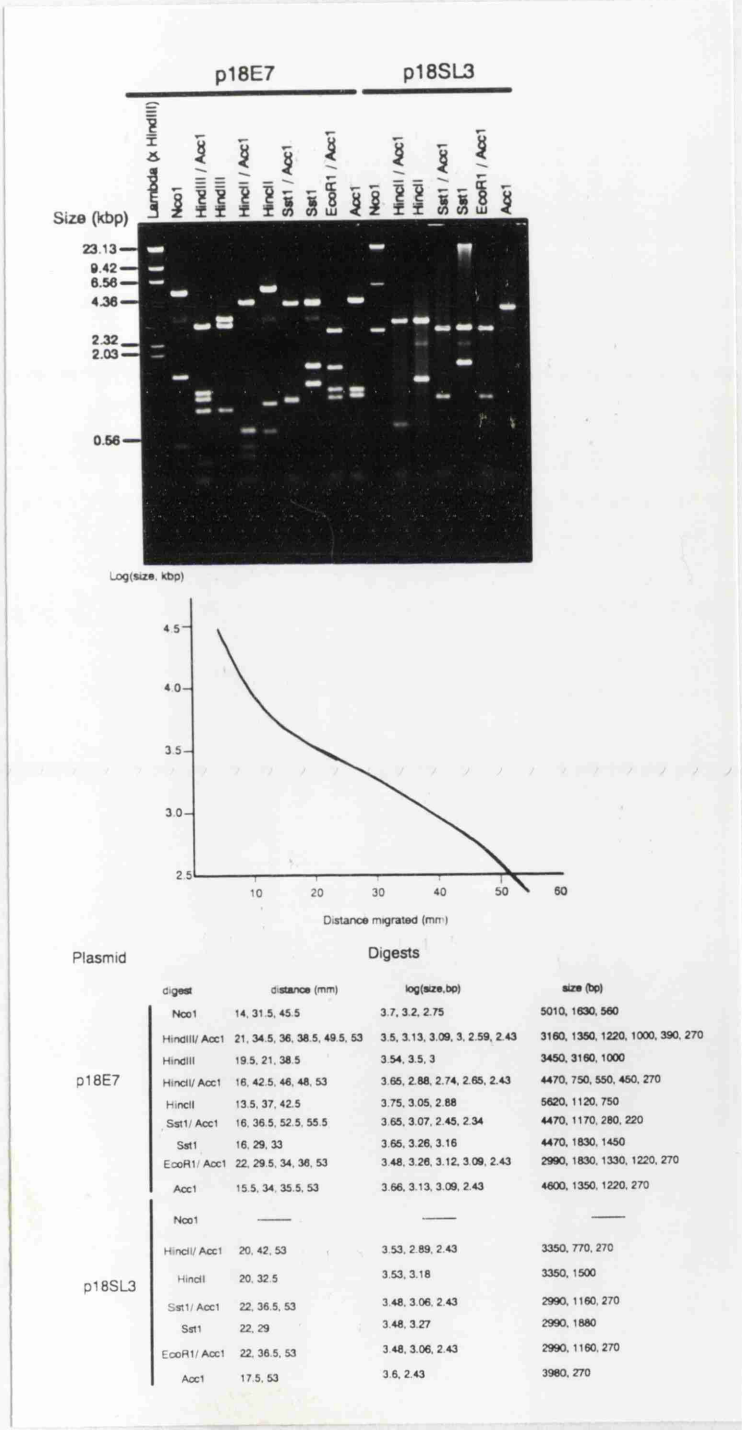


Digests

Plasmid	digest	distance (mm)	log(size,bp)	size (bp)
p18E7	HindIII/EcoR1	22, 25, 43.5, 57.5, 58.5	3.5, 3.43, 3.01, 2.34, 2.28	3160, 2660, 1030, 220, 190
	HindIII/Nco1	23.5, 35.5, 43.5, 50, 52	3.46, 3.19, 3.01, 2.79, 2.7	2900, 1540, 1030, 620, 500
	HindIII	22, 23.5, 43.5	3.5, 3.46, 3.01	3160, 2900, 1030
	EcoR1/Nco1	25, 31.5, 35.5, 51.5	3.42, 3.27, 3.19, 2.72	2660, 1880, 1540, 530
	HincII/Nco1	17, 42, 50, 51.5, 55	3.65, 3.06, 2.79, 2.72, 2.53	4470, 1140, 620, 530, 340
	HincII	14.5, 42, 48.5	3.73, 3.06, 2.85	5390, 1140, 710
	Sst1/Nco1	17.5, 46.5, 51, 55	3.64, 2.92, 2.75, 2.53	4340, 840, 560, 340
	Sst1	17.5, 33, 37.5	3.64, 3.24, 3.14	4340, 1750, 1390
p18SL3	EcoR1/Nco1	23, 46.5, 51.5	3.48, 2.92, 2.72	2990, 840, 530
	HincII/Nco1	23, 51.5, 55	3.48, 2.72, 2.53	2990, 530, 340
	HincII	23, 37	3.48, 3.15	2990, 1410
	Sst1/Nco1	25, 46.5, 51.5, 55	3.42, 2.92, 2.72, 2.53	2660, 840, 530, 340
	Sst1	25, 33.5	3.42, 3.23	2660, 1700

Figure 8.4: Restriction enzyme mapping of MIP1α gene-containing genomic-subclone plasmid p18E7 and its derivative p18SL3.

5 μg aliquots of p18E7 or p18SL3 plasmid DNA were digested with a panel of restriction enzymes, alone or in combination, as shown above the photograph. The digested DNA was electrophoresed on a 1.2% (w/v) agarose gel and stained with ethidium bromide. Molecular weight markers (λxHindIII) electrophoresed adjacent to the plasmid DNA samples are shown. Distance migrated by the molecular weight markers and plasmid restriction fragments was measured, and the size (in bps) of each restriction fragment calculated, as shown below the photograph.

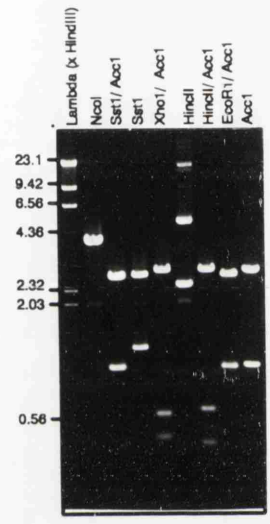


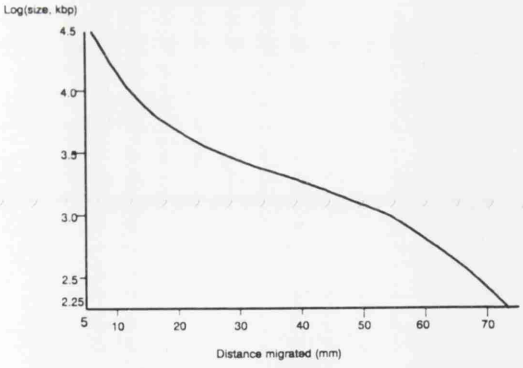
Plasmid Digests

	digest	distance (mm)	log(size,bp)	size (bp)
p18E7	Nco1	14, 31.5, 45.5	3.7, 3.2, 2.75	5010, 1630, 560
	HindIII/ Acc1	21, 34.5, 36, 38.5, 49.5, 53	3.5, 3.13, 3.09, 3, 2.59, 2.43	3160, 1350, 1220, 1000, 390, 270
	HindIII	19.5, 21, 38.5	3.54, 3.5, 3	3450, 3160, 1000
	HincII/ Acc1	16, 42.5, 46, 48, 53	3.65, 2.88, 2.74, 2.65, 2.43	4470, 750, 550, 450, 270
	HincII	13.5, 37, 42.5	3.75, 3.05, 2.88	5620, 1120, 750
	Sst1/ Acc1	16, 36.5, 52.5, 55.5	3.65, 3.07, 2.45, 2.34	4470, 1170, 280, 220
	Sst1	16, 29, 33	3.65, 3.26, 3.16	4470, 1830, 1450
	EcoR1/ Acc1	22, 29.5, 34, 36, 53	3.48, 3.26, 3.12, 3.09, 2.43	2990, 1830, 1330, 1220, 270
	Acc1	15.5, 34, 35.5, 53	3.66, 3.13, 3.09, 2.43	4600, 1350, 1220, 270
p18SL3	Nco1	——	——	——
	HincII/ Acc1	20, 42, 53	3.53, 2.89, 2.43	3350, 770, 270
	HincII	20, 32.5	3.53, 3.18	3350, 1500
	Sst1/ Acc1	22, 36.5, 53	3.48, 3.06, 2.43	2990, 1160, 270
	Sst1	22, 29	3.48, 3.27	2990, 1880
	EcoR1/ Acc1	22, 36.5, 53	3.48, 3.06, 2.43	2990, 1160, 270
	Acc1	17.5, 53	3.6, 2.43	3980, 270

Figure 8.5: Restriction enzyme mapping of plasmid p18S2.

5 μg aliquots of DNA from plasmid p18S2, a derivative of MIP1α gene-containing plasmid clone p18E7, were digested with a panel of restriction enzymes, alone or in combination, as shown above the photograph. The digested DNA was electrophoresed on a 1.2% (w/v) agarose gel and stained with ethidium bromide. Molecular weight markers (λxHindIII) electrophoresed adjacent to the plasmid DNA samples are shown. Distance migrated by the molecular weight markers and plasmid restriction fragments was measured, and the size (in bps) of each restriction fragment calculated, as shown below the photograph.





Digests

digest	distance (mm)	log(size,bp)	size (bp)
Nco1	22	3.61	4100
Sst1/ Acc1	31, 50, 76	3.49, 3.08, 2.08	2700, 1200, 120
Sst1	31, 46	3.43, 3.16	2700, 1440
Xho1/ Acc1	29.5, 59.5, 64	3.45, 2.83, 2.66	2820, 670, 460
HincII	--------	--------	--------
HincII/ Acc1	29.5, 58.5, 65.5	3.45, 2.85, 2.8	2820, 710, 400
EcoR1/ Acc1	31, 50, 76	3.49, 3.08, 2.08	2700, 1200, 120
Acc1	29.5, 50	3.45, 3.08	2820, 1200

Several restriction fragments derived from p18E7, p18SL3 and p18S2 were subcloned into the pUC18 mcs, as shown in figure **8.6** and Table **6.1**, and the M13 Universal- and M13 Reverse primers were used to partially sequence these subclones. From the sequences obtained from these experiments, single-stranded oligonucleotide sequencing primers were designed and used for further sequencing, and from these experiments, yet further sequencing primers were designed and used. The sequences of these primers, MOL-1 to MOL-7, are shown in Table **6.1**, and the approximate sites of priming recognised by these oligonucleotides in the described pUC18 plasmid subclones, are shown in figure **8.6** (MOL-1 equals primer 1, etc.). A further two single-stranded oligonucleotide primers, 5496 and 5498 (figure 6, and see Table **6.1** for sequences), designed from the MIP1α gene cDNA sequence, and therefore recognizing MIP1α gene coding sequences (donated by Dr.S.Wolpe, Genetics Institute, Cambridge, MA, USA), were also used in the sequencing strategy, which is summarized in figure **8.6**.

All sequences obtained were subsequently confirmed by sequencing over the same region in the opposite direction. The full sequence of the MIP1α gene, showing relevant restriction sites and including 350 bp of 5'flanking sequence and ~230 bp of 3'flanking sequence is shown in figure **8.7**, and the results of this study were published in Nucleic Acids Research (Grove *et al*, 1990), and were assigned EMBL Genebank accession number X53372.

CHAPTER 9: CHARACTERISTICS OF MIP1α GENE EXPRESSION.

9.1 THE MIPα GENE IS AN EARLY-RESPONSE GENE IN MURINE MACROPHAGE CELL-LINES.

9.1.1 Overview.

As outlined in the Introduction, the MIP1α may be a hemopoietic cell-specific early response gene. As many early-response genes are rapidly induced by serum in

Figure 8.6: Restriction map of MIP1α gene-containing genomic-subclone plasmid p18E7 and summary of the strategy used to sequence the MIP1α gene.

Summary of the DNA restriction fragments derived directly or indirectly (by further sub-cloning) from Lambda clone 1141, and subcloned into the mcs of plasmid pUC18. Top of figure: the ~4.4 kbp MIP1α gene-containing EcoR1 restriction fragment E7 is shown, with a summary (to scale) of the restriction enzyme analysis of the MIP1α gene locus; several Acc1 sites are not shown, for reasons stated in the text. The region of the p18E7 plasmid DNA that contains the MIP1α gene is indicated by the blue-shaded box. Yellow boxes: restriction fragments derived directly or indirectly from plasmid p18E7:- SL3: large Sst1 fragment derived from p18E7; S2: small Sst1 fragment derived from p18E7; L4: large HincII fragment derived from p18E7; HII: small HincII fragment derived from p18E7; NE1: small Sst1-Nco1 fragment derived from p18SL3; SL3NS: large Sst-Nco1 fragment derived from p18SL3; N4: Nco1 fragment derived from p18SL3; LS2Acc: large Acc1 fragment derived from p18S2; SS2Acc: Sst1-Acc1 fragment derived from p18S2. Single-stranded oligonucleotide primers which were used to sequence these subcloned fragments are denoted by open boxes, each containing the name of the relevant primer, and are positioned above the approximate position of the sequence recognized by that primer. Arrows denote the direction of sequencing by each primer. 1 to 7: primers MOL-1 to MOL-7, respectively.

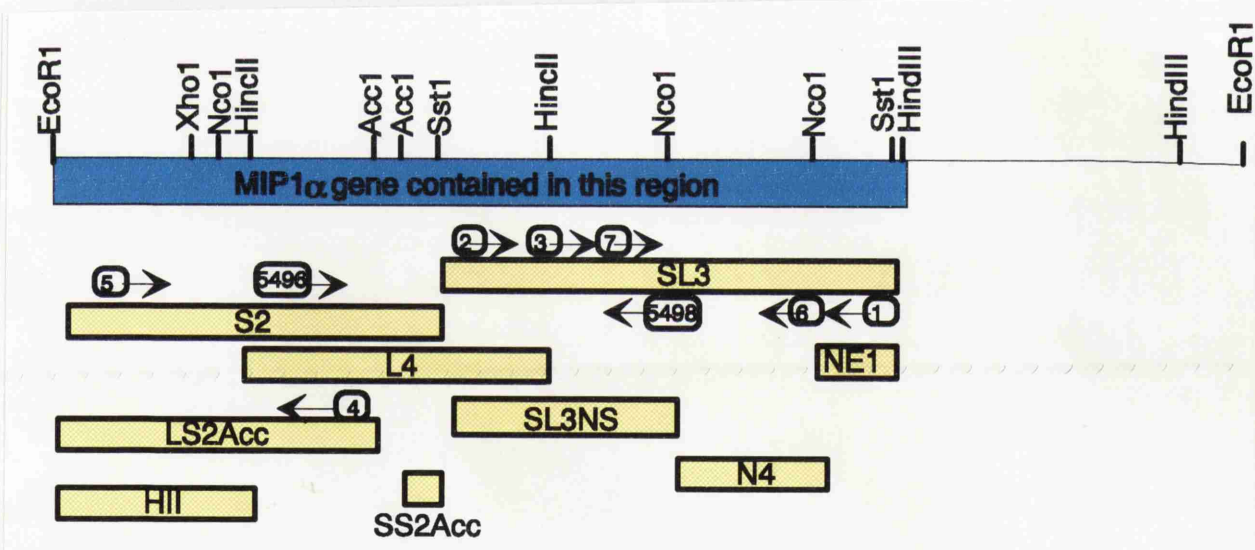


Figure 8.7: Sequence of the MIP1α gene.

The MIP1α gene was sequenced in both directions as described in the text. Numbers above the sequence denote nucleotide position relative to the cap site (+1), and the sequence shown is from -350 bps to +1980 bps. Exons are in bold italic typeface, and intron- and 5' and 3' flanking sequences are in normal typeface. START (+81 bp) and STOP (+1077 bp) denote the translational start and stop sites respectively. The 3' untranslated region (3' UTR) ATTTA repeats referred to in the text are underlined. Marked sequences denote the following:- +1115 (Nco1) and +1640 (Nco1): 5' and 3' ends, respectively, of N4 restriction fragment described in the text; -350 bp (Nco1), -270 bp (HincII), -220 bp (pM-220), -190 bp (pM-190) and -160 bp (EcoRV): 5' ends of MIP1α promoter constructs pM-350, pM-270, pM-220, pM-190 and pM-160, respectively; +36 bp: 3' end of MIP1α promoter constructs; -18 bp (Nde1): Nde1 restriction site used in verification of sizes of mutant pM-190 MIP1α promoter constructs (chapter **13**); -270 bp (HincII) and +393 bp (Sst1), +393 bp (Sst1) and +713 bp (HincII), +713 bp (HincII) and +1320 bp (Xmn1): 5' and 3' ends of restriction fragments comprising nuclear run-on probes MNR1, MNR2 and MNR3, respectively (chapter **15**).


-350
NcoI
CCATGGCAAGGAATGGGTTTGGAGTGTTTATTCACAGAACGGTTCTCATGA
-270
HincII
GATGGGACCAGCTAAGAATAGCCCTGGGTTGACACTGTCCTACCTCCTCCT
-220
pM-220
GCTCATAAGAGAAACTACTTCCCCACAAGAAGAAAGAATAGGTCACGAGTT
-190
pM-190
-160
EcoRV
GAGAGCTGAGACTTATATCTCAGAGATGCTATTCTTAGATATCCTGGGCCC
CTGTGGTCACTGTGGACCCTGGGTTGTGTAATATCCATCATGACACCATTGC
TGTGCTTAAAATTTTCCCTCCTCACCCCCAGATTCCATTTCCTCATCTGCTA
-18
NdeI
+1
GGGCTACCTATAAGAGAAGGGGCATATGACTTCAGACACCAAGAAGGATACAA
+36
GCAGCAGCGAGTACCAGTCCCTTTTCTGTTCTGCTGACAAGCTCACCCTCTG
+81
START
TCACCTGCTCAACATCATGAAGGTCTCCACCACTGCCCTTGCTGTTCTTCTC
TGTACCATGACACTCTGCAACCAAGTCTTCTCAGCGCCATGTGAGTCTACAC
TGCTACAGGAGGTGTCCCCAGTCTCTGGTCTAAGGCAGACCATAGCAGTCTT
TTCTGTGGTTTCAGAGTCTACAAGAGAAAAGAAGCTGGTCTTTCTCTTTGAG
ATTTTTATTTTCCTCTCTATGGTGTGGAAGGGTCTAACCTTTTATAGGTACC
CAGAACTTGAAATCACAGTAAGGATAGTGTTGTCATGTACTTACTAGCCAGC
+393
SstI
CCTGGGATTCTTAAGGAGCTCTGGTGTCTTCTTTGCTGACTTCTGGTATTAG
AGAAAGCTGTGGGAAAGACACAGGGCAACACATAGAGAAAGAGAAAGCCCAC
AGTGAAAATATAGATAGGACAGAGACAGAAACAATGACATTTCTGGTCCAGA
TGCCCAAATGCCTTCAGATCCTTAGCCTTCCAACTCCACCCCAAGCACCTTC
CTCGCCTCTTAAGTTGTTTTAGGTGTCCTACCCTGCTCAATGATGACTTTCT
TCCCCTACAGATGGAGCTGACACCCCGACTGCCTGCTGCTTCTCCTACAGCC
+713
HincII
GGAAGATTCCACGCCAATTCATCGTTGACTATTTTGAAACCAGCAGCCTTTG
CTCCCAGCCAGGTGTCATGTAAGTTCTGTTCTCGTGCTTTTCCTGATAAAGT
GGGGTGAGGAACAGTGTAAGAGAGGGTTTCTCAGCTCGTAGAGAGTGGGGGC
GGGGGGGGCAGTCACAAAAAGGGAAGCACAGGATCATCTAAAGGGTGCCAGT
GAGAGGACGGGAGCGATGGAGAAACCACAGAGGAAGTCAGACTCTCCCATTG
GATATTTAAGTTTTTTGACCCTTACCTCTCTCTGTGTAGTTTCCTGACTAAG
AGAAACCGGCAGATCTGCGCTGACTCCAAAGAGACCTGGGTCCAAGAATACA
+1077
STOP
TCACTGACCTGGAACTGAATGCCTGAGAGTCTTGGAGGCAGCGAGGAACCCC
+1115
NcoI
CCAAACCTTCCATGGGTCCCGTGTAGAGCAGGGGCTTGAGCCGAACATTCCT
GCCACCTGCATAGCTCCATCTCCTATAAGCTGTTTGCTGCCAAGTAGCCACA
TCGAGGGACTCTTCACTTGAAATTTTATTTAATTTAATCCTATTGGTTTAAT
ACTATTTAATTTTGTAATTTATTTTATTGTCATACTTGTATTTGTGACTATT
+1320
XmnI
TATTCTGAAAGACTTCAGGACACGTTCCTCAACCCATCCCTCCAGTTGGTCA
CACTGTTGGTGACAGCTATTCCTAGGTAGACATGATGACAAAGTCATGAACT
GACAAATGTACAATGGATGCTTTGTTTATACCAGAGAAGTAATAAATATGCC
CTTTAACAAGTGACTGGTGTTGAGTTTTATTTTATTTTATTTTTTATTTTTA
TTTTTCTGGAAAGTCAGACTTGCTGGTTAAGAGGAATAACAGCAAGGAATAG
GAAGTCGTGAATGGAAGTTGGTAGTGTGTTGGAAGGGGTGGGGAGAGGAGCA
+1640
NcoI
TGAGGAGACTCCCATGGAGTAGACTTCTCGACTTCATAATGGAAATATGGAT
CTTTGGAGACTGGTAAAGGAATAAGCCAGGCAAACAAATATACTACCAAGGG
GCA

both continuously dividing and serum-starved NIH 3T3 fibroblast cells (Lan *et al*, 1985; Cortner *et al*, 1991), I examined the possibility that serum might induce MIP1α mRNA accumulation in macrophages. Because rapid accumulation of early-response gene mRNA can also be triggered by "stress" of the cells, for example *c-fos* mRNA rapidly accumulates in cells surrounding a line scratched between cells on the bottom of a tissue-culture flask (Gillespie, 1991), in experiments investigating the effect of fresh serum on MIP1α mRNA accumulation in macrophages, I included controls that rule out induction of MIP1α mRNA simply by manipulation of the cells.

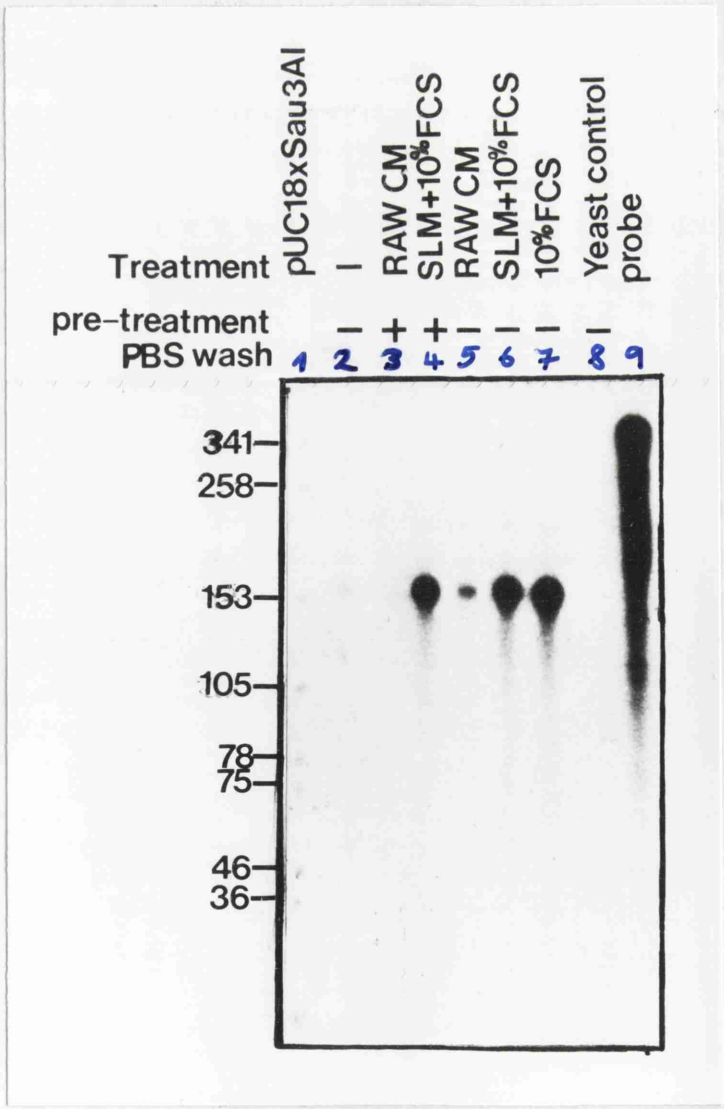
9.1.2 Rapid induction of MIP1α mRNA in RAW 264.7 cells by fresh 10% (v/v) FCS.

MIP1α mRNA levels in RAW 264.7 cells were initially examined in the rapid RNase protection assay. A length of DNA containing 220 bps of the MIP1α proximal promoter plus the 156 bps MIP1α first exon-encoding sequences, was generated by PCR and subcloned into the pSK(-) plasmid, generating plasmid pSK-MIP1α (figure **10.7a** and Tables **6.1** and **6.4**). This plasmid, designed to detect correctly-initiated MIP1α RNA, was used to generate [^{32}P-α]-UTP-labelled antisense MIP1α RNA probe, and a large excess of this probe was incubated with total RNA from RAW 264.7 cells then subjected to limited RNaseA/T1 treatment, both of whose substrate is single-stranded-, but not double-stranded RNA, in an RNase protection assay.

After replating RAW 264.7 cells in medium containing fresh 10% (v/v) FCS, and incubating the cells for 3 days, a low level of MIP1α mRNA is detectable by RNase protection (figure **9.1**, lane 2). These cells are hereby defined as "unstimulated RAW 264.7 cells". If the medium present on the unstimulated RAW 264.7 cells ("RAW cell conditioned medium" (RAW CM, see chapter **7** section **7.1.5**) is removed, and the cells are PBS-washed before adding the RAW CM back to the cells, MIP1α mRNA levels are actually reduced compared to basal levels (compare lanes 2 and 3, figure **9.1**). If the RAW CM is simply removed then put back onto the cells without PBS-washing of the cells, a moderate induction of MIP1α mRNA levels is observed (figure **9.1**, lane 5). The greatest induction observed in this experiment is when fresh 10% (v/v) FCS is added directly to the unstimulated cells (lane 7).

Figure 9.1: Effect of fresh 10% FCS on MIP1α gene expression in RAW 264.7 macrophage cell-line

RNase protection analysis of total RAW 264.7 cellular RNA. RAW 264.7 cells were incubated for 3 days after replating in SLM containing fresh 10% (v/v) FCS, then variously pre-treated prior to various experimental treatments for 3 hours:- Lane 2: no treatment control; Experimental pre-treatments were as folllows:- Lanes 3-7, RAW CM removed from flasks and stored; lanes 3 and 4, cells PBS-washed *in-situ*; lanes 5, 6, 7, no further pre-treatment. Experimental treatments were as follows:- Lanes 3 and 5, cells incubated in RAW CM; Lanes 4 and 6, cells incubated in fresh SLM containing fresh 10% (v/v) FCS; Lane 7, cells incubated in RAW CM containing additional fresh 10% (v/v) FCS. Total RNA was then prepared, and 20 μg hybridized with a uniformly [α-^{32}P]-UTP-labelled 415-base antisense MIP1α RNA riboprobe (synthesized from linearized pSK-MIP1α plasmid template: figure 16) at 45^{o}C for 24 hours in an RNase protection assay. Hybrids, and 20 μg of yeast RNA control, were then digested with an RNaseA/RNaseT1 mixture (5μg/ml and 100U/ml respectively) and protected RNA fragments resolved by denaturing PAGE and autoradiography. Lane 1: molecular weight markers: plasmid pUC18 restriction digested with Sau3AI and 5' end-labelled using [γ-^{32}P]-ATP and polynucleotide kinase; lane 8, yeast control RNA; lane 9, yeast control RNA hybridized with uniformly [α-^{32}P]-UTP-labelled 415-base antisense MIP1α RNA riboprobe (synthesized from linearized pSK-MIP1α plasmid template) and mock-digested.



The conclusion from this experiment is that MIP1α mRNA rapidly accumulates in RAW 264.7 cells specifically in response to **fresh** 10% (v/v) FCS; it is evident that a similar level of induction of MIP1α RNA by fresh 10% (v/v) FCS is obtained in the presence of either RAW CM or of fresh SLM. A further point that can be inferred from this experiment is that active serum growth factor(s) may be present in low amounts in the flasks containing uninduced cells but are not presented correctly to the cells; removal and re-addition of the RAW CM presumably disturbs these growth factor(s) and allows them to become available to the cells (compare lane 2 to lane 5, figure **9.1**).

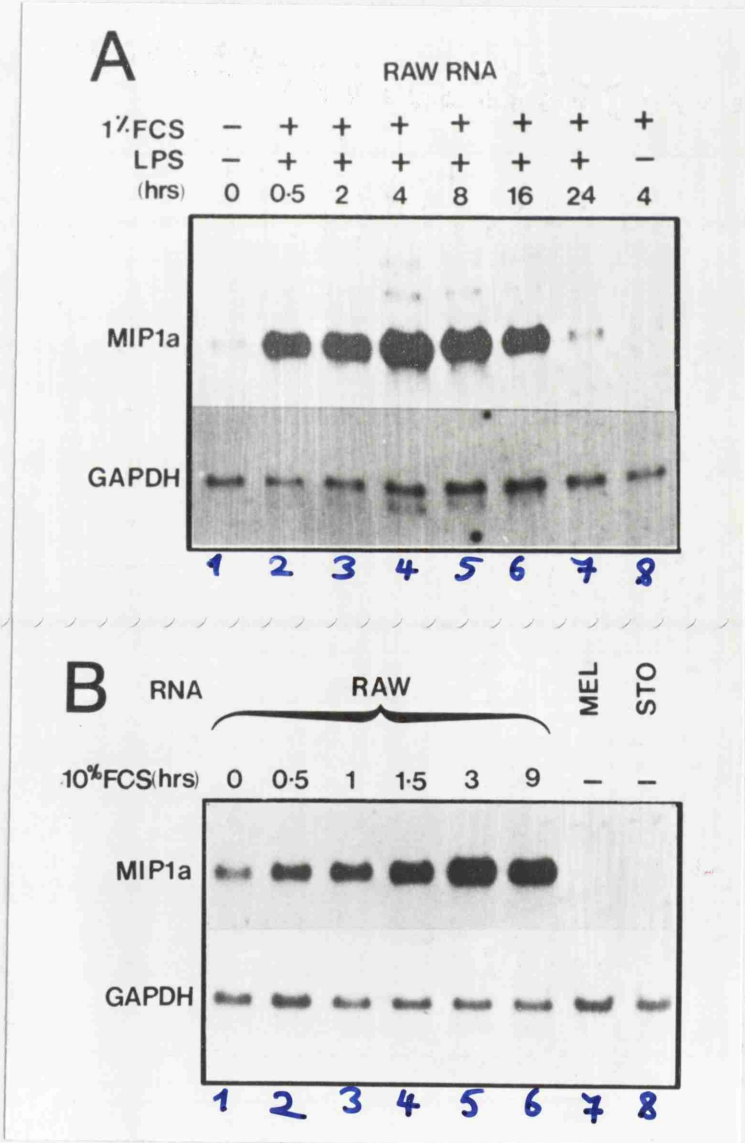
9.1.3 Rapid and transient induction of MIP1α mRNA by fresh serum and LPS in RAW 264.7 cells.

Northern blotting of total RNA prepared from unstimulated RAW 264.7 cells confirms that low but readily-detectable levels of MIP1α mRNA are present in these cells (figures **9.2a** and **9.2b**, lanes 1). In later experiments investigating the functional activity of MIP1α promoter sequences, it was noted that the promoter response to LPS was maximal in the presence of fresh 1% (v/v) FCS (see chapter **10**, section **10.5**), but that MIP1α promoter activity was not enhanced by fresh 1% (v/v) FCS alone. Hence, I investigated the accumulation of MIP1α mRNA in RAW 264.7 cells stimulated with 2 μg/ml LPS in the presence of fresh 1% (v/v) FCS.

In the experiment shown in figure **9.2a**, 2 μg/ml of LPS in the presence of fresh 1% (v/v) FCS was added directly to RAW 264.7 cells growing in RAW CM, the cells were incubated for the indicated times, and total RNA was extracted. As shown in figure **9.2a**, on addition of 2 μg/ml of LPS plus fresh 1% (v/v) FCS to RAW 264.7 cells growing in RAW CM, MIP1α mRNA is induced over 10-fold within thirty minutes, peaks at about 30-fold over uninduced levels at ~3-6 hours after induction, and returns to levels only about 2-fold greater than basal- by 24 hours after induction. In contrast, addition of fresh 1% (v/v) FCS (minus LPS) for four hours to unstimulated RAW 264.7 cells resulted in no induction over basal levels of MIP1α mRNA (figure **9.2a**,lane 8).

Figure 9.2: Cell-specific and transiently inducible MIP1α gene expression.

Northern blot analysis of 20 μg of total cellular RNA. Filters were first probed with an [α-^{32}P]-UTP-labelled MIP1α antisense RNA riboprobe (synthesized from linearized pSK-MIP-cDNA plasmid template), then stripped and reprobed with an [α-^{32}P]-dCTP-labelled random-primed rat GAPDH cDNA probe as a loading control. RAW 264.7 cells were replated in SLM containing fresh 10% (v/v) FCS and incubated for 3 days prior to the experiment, ie. at the start of the experiment, all RAW 264.7 cells were in RAW CM. **A:** Total RNA prepared from RAW 264.7 cells in RAW CM (lane 1, 0 hrs), or incubated in RAW CM containing fresh 1% (v/v) FCS (+) in the presence or absence (+/-) of 2 μg/ml of LPS for the times indicated (hrs). **B:** Total RNA prepared from MEL or STO cells (as indicated above the autoradiograph), from RAW 264.7 cells in RAW CM (lane 1, 0 hrs), or from RAW 264.7 cells from which RAW CM was removed, and which were then incubated in SLM containing fresh 10% (v/v) FCS for the times indicated.



To investigate the kinetics of the serum-responsiveness of the MIP1α gene in RAW 264.7 macrophages, the medium was removed from unstimulated RAW 264.7 cells, replaced with medium containing fresh 10% (v/v) FCS, and total RNA was isolated from these cells over a timecourse. As shown in figure **9.2b**, three hours after the addition of medium containing fresh 10% (v/v) FCS, MIP1α mRNA was approximately 20-fold more abundant than in unstimulated RAW 264.7 cells, and by nine hours after stimulation, MIP1α mRNA levels were down to half those present at three hours after stimulation.

These experiments (figures **9.2a** and **9.2b**) show that endogenous MIP1α mRNA is rapidly and transiently induced in RAW 264.7 cells by both 2 μg/ml of LPS and by fresh 10% FCS (though the induction is slightly less rapid by fresh 10% (v/v) FCS compared to LPS). As accumulation of MIP1α mRNA is also rapidly induced by cycloheximide in RAW 264.7 cells (M.Plumb, personal communication), MIP1α is therefore an early-response gene in these cells.

9.2 CELL-SPECIFIC EXPRESSION OF MIP1α mRNA.

The MIP1α gene is reportedly cell-specifically expressed in a subset of hemopoietic cell-types, so to investigate possible mechanisms for such cell-specific expression of the MIP1α gene, I therefore screened non-macrophage cell-lines for the presence of MIP1α mRNA, using Northern blot analysis of total cellular RNA. As shown in figure **9.2b** (lanes 7 and 8), neither continuously cyling embryonic fibroblast STO cells or F4-12B mouse erythroleukaemia (MEL) cells express detectable MIP1α mRNA. Further studies in this laboratory (M.Plumb, personal communication), using the polymerase chain reaction (PCR), of cDNA, for detection of low levels of MIP1α mRNA sequences, confirmed that MIP1α mRNA is undetectable in either STO or MEL cells, or in mouse epidermal keratinocyte MEK 79 cells, but is present in J774.2 macrophages and in WEHI myeloid progenitor cells (P.Daubersies, personal communication). These observations are consistent with the cell-restricted expression of MIP1α reported by others (Brown *et al*, 1989; Burd *et al*, 1989; Martin *et al*, 1991; Mtsue *et al*, 1992; Obaru *et al*, 1986; Zipfel *et al*, 1989). I

therefore used STO and MEL cells as control cells in subsequent experiments (see below).

9.3 INVESTIGATION OF THE CHROMATIN CONFORMATION OF THE MIP1α GENE LOCUS IN MACROPHAGES AND CONTROL CELLS BY NUCLEAR DNase1 HYPERSENSITIVITY STUDIES.

9.3.1 The MIP1α gene proximal promoter region is DNase1 hypersensitive in macrophage cell lines J774.2 and RAW 264.7.

As outlined in the Introduction, active- or potentially active ("primed") genes are associated with DNase1 hypersensitive sites, while the DNA of chromatin in the loci of genes not active in a particular cell type, is comparatively resistant to digestion by DNase1. Therefore, in order to determine whether the MIP1α gene is in an active-, and hence inducible-, nuclear conformation in non-expressing cells, and to map gene regulatory sequences associated with cell-specific nuclear DNase1 hypersensitive sites *in-vivo*, the nuclear conformation of the MIP1α gene was examined in various cell-types.

In experiments carried out in this laboratory, nuclei from unstimulated macrophage cells (J774.2) were digested with increasing concentrations of DNase1, genomic DNA was isolated, digested with HindIII or EcoR1, resolved by gel electrophoresis, Southern blotted, and probed with a uniformly-labelled MIP1α cDNA antisense RNA riboprobe generated from the pSK-MIPcDNA plasmid (Table **6.1**).

The HindIII analysis revealed an ~ 7 kbp parental band (figure **9.3a**), as expected from the restriction map of the MIP1α gene locus (figure **8.2**), and also a 2.1-2.3 kbp subgenomic doublet with increasing nuclear DNase1 digestion (figure **9.3a**), which is due to an ~250-350 bp region of *in-vivo* DNase1 hypersensitivity of the MIP1α gene locus in J774.2 cells, situated between the two HindIII restriction enzyme recognition sequences shown in figure **8.2**, and located ~2.1-2.3 kbps from one of them. A doublet (as seen in figure **9.3a**), rather than single-banded, region of DNase1 hypersensitivity signifies that a nucleosome(s) has been displaced from the DNA site

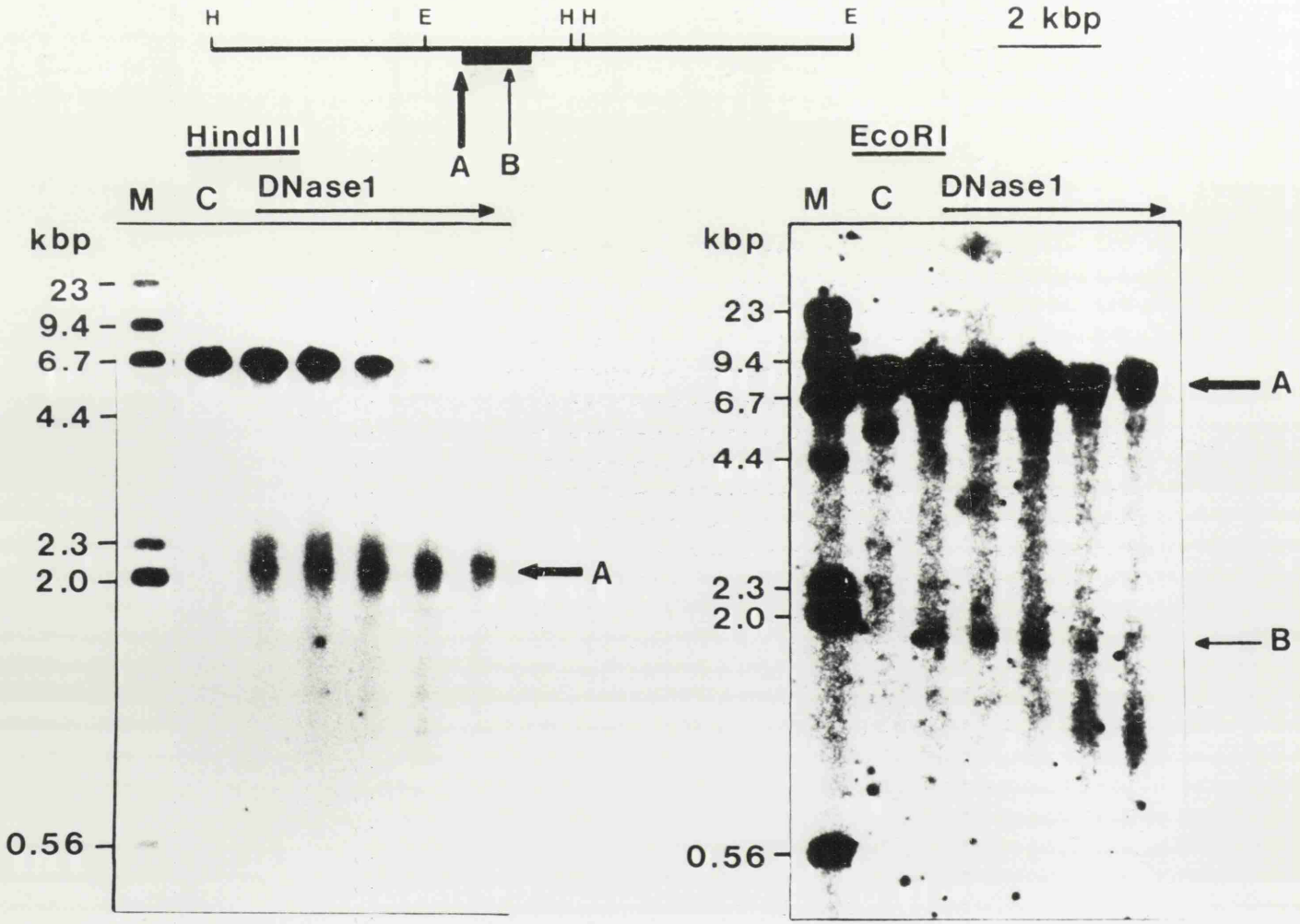

H
E
H H
E
2 kbp
A
B
HindIII
M
C
DNase1
kbp
23
9.4
6.7
4.4
2.3
2.0
0.56
A
EcoRI
M
C
DNase1
kbp
23
9.4
6.7
4.4
2.3
2.0
0.56
A
B

Figure 9.3: Chromatin configuration of the MIP1α gene locus in the J774.2 macrophage cell-line.

Southern blot analysis of DNA (10 μg) prepared from nuclei of unstimulated J774.2 cells which had been digested with increasing concentrations of DNase1. DNA was digested with either HindIII or EcoR1 restriction enzyme (as shown above the corresponding autoradiograph), transferred to nitrocellulose and probed with a uniformly [α-^{32}P]-UTP-labelled MIP1α antisense RNA riboprobe (synthesized from linearized pSK-MIP-cDNA plasmid template). Lane M, molecular weight markers (λ DNA digested with HindIII then 5' end-labelled using [γ-^{32}P]-ATP and polynucleotide kinase): the size of the markers is shown to the left of each autoradiograph; lane C, control DNA from J774.2 cell nuclei that had not been treated with DNase1. The subgenomic bands indicative of DNase1 hypersensitive sites A and B are marked marked by arrows to the right of each autoradiograph. The MIP1α gene locus parental HindIII and EcoR1 restriction fragments are ~6.7 kbps and ~9 kbps in size, respectively, as seen on the respective autoradiographs. A genomic restriction map of the MIP1α gene locus in J774.2 cells, showing EcoR1 (E) and HindIII (H) recognition sites and the location of the DNase1 hypersensitive sites A and B (denoted by arrows) is shown in cartoon form above the autoradiographs, with the MIP1α gene shown as a closed box and the scale of the cartoon (in kbps) as shown. **This figure is reproduced by kind permission of Dr. M.Plumb.**

in question, and the central region of the site is occupied by transcription factors, while the DNA sequences immediately flanking the site of transcription factor binding are not bound by proteins and are therefore hypersensitive to digestion by DNase1.

The EcoR1 analysis revealed a parental band of ~8.8 kbp (figure **9.3b**) as expected (figure **8.2a**), and a sub-genomic band that almost co-migrates with the parental band (figure **9.3b**), so confirming the position of the major region of DNase1 hypersensitivity described above as being over the MIP1α gene proximal promoter region. Also in the EcoR1 analysis, a weaker faster-migrating sub-genomic band of ~1.8 kbp was observed with increasing DNase1 digestion (figure **9.3b**), which corresponds to a region of weak DNase1 hypersensitivity which maps approximately to the second intron of the MIP1α gene (figures **8.6** and **8.7**), but this second region of DNase1 hypersensitivity is very weak and may not be significant; a possible reason for its presence (nucleosome positioning) is addressed in the Discussion.

A similar analysis was performed on nuclei isolated from unstimulated RAW 264.7 cells, and from 12-hour LPS-stimulated RAW 264.7 cells (figure **9.4**). Although the data presented is of poor quality, it is evident that HindIII restriction enzyme digestion of DNase1-treated nuclei isolated from either unstimulated or 12 hour LPS treated RAW 264.7 cells reveals a 2.1-2.3 kbp sub-genomic doublet. Hence, similar MIP1α proximal promoter DNase1 hypersensitivity is present in unstimulated J774.2 and RAW 264.7 cells, and 12 hour LPS stimulation of the latter cells does not result in the appearance of any additional DNase1 hypersensitive sites within a 5 kbp flanking region 5' of the MIP1α gene (figure **9.4**).

9.3.2 The MIP1α proximal promoter DNase1 hypersensitive region is absent in non-myeloid cell lines.

The nuclear conformation of the MIP1α gene in non-macrophage cell lines was also investigated in this laboratory (Plumb *et al*, 1991; M.Plumb, personal communication). The MIP1α proximal promoter DNase1 hypersensitive region was present in the myeloid progenitor WEHI cell line, but no MIP1α gene locus hypersensitive sites were detected in STO- or MEK79 cells, although a c-Harvey Ras

gene promoter DNase1 hypersensitive site was detected using the same STO cell nuclei (Plumb *et al*, 1991). Hence, the endonuclease accessibility of the MIP1α gene is tissue-specific, and correlates with the detection of MIP1α mRNA in macrophage cell-lines and in non-MIP1α gene-expressing control cells.

CHAPTER 10: MIP1α GENE PROMOTER FUNCTIONAL STUDIES.

10.1 OVERVIEW.

Results presented in chapter **9** suggested that, within an ~10 kbp locus containing the MIP1α gene, the MIP1α proximal promoter region is the major region functionally important for macrophage-specific MIP1α gene expression, an assertion that is further supported by the high conservation of this 5' flanking region between murine and human MIP1α genes (figure **11.1**).

Although these results cannot exclude the possibility that more distant *cis*-acting gene regulatory elements, for example putative enhancers and/or locus control regions (LCRs) (see Introduction), are important in the regulation of transcription of the MIP1α gene in macrophages or other cell types, the obvious and easiest next step was to examine the activity of the MIP1α promoter in macrophages and control cells, using functional cell transfection assays of MIP1α promoter function.

Functional cell transfection assays of promoter function, as outlined in the Introduction and Methods sections, involve linking of test gene (ie. MIP1α) promoter fragments to a reporter gene, transfection of the resulting plasmid DNA construct into different mammalian cell types, and assaying for reporter gene expression in the cells or surrounding cell growth medium. Analysis of MIP1α promoter function in such assays would answer several points: 1) If the proximal MIP1α promoter sequences are the major determinant of MIP1α gene transcriptional activity within an ~10 kbp locus surrounding the gene, as suggested by the *in-vivo* DNase1 hypersensitivity studies, it would be expected that additional MIP1a promoter sequences would not

contribute further activity, in functional transfection assays, to MIP1a promoter constructs containing only the proximal MIP1α promoter sequences; 2) Is the proximal MIP1α promoter sufficient to confer macrophage cell-specific transcriptional activity on a reporter gene; 3) As endogenous MIP1α gene RNA levels are modulated in macrophages in response to cell stimuli such as LPS and fresh 10% FCS, does the MIP1α proximal promoter contain *cis*-regulatory elements that are responsive to these agents in macrophages ? Furthermore, as the same, or overlapping *cis*-regulatory elements are involved in the regulation of basal, induced and repressed transcription of early-response genes (see chapter **3**, section **3.3.5**), identification of MIP1α promoter regulatory elements in functional transfection experiments would pinpoint promoter regions that are likely to be involved in the regulation of MIP1α gene transcription in macrophages and/or other cell types in response to external stimuli other than LPS or fresh 10% FCS.

10.2 SYNTHESIS OF MIP1α PROMOTER DELETION CONSTRUCTS.

In order to try to ensure that reporter gene transcripts would initiate at the same sequence as endogenous MIP1α transcripts, all MIP1α promoter deletion constructs contained the MIP1α gene cap site and its immediate flanking sequences. Also, as the sequence of the TATA box, and its immediate flanking sequences, are important determinants of communication between the basal promoter and the promoter/enhancer of a gene (see chapter **3**), all MIP1α promoter deletion constructs contain the MIP1α TATA box and basal promoter.

Because the region of the endogenous MIP1α gene 5' untranslated region immediately downstream of the cap site contains no appropriate restriction enzyme recognition sites (see figure **8.7**), MIP1a promoter deletions pM-900 and pM-270 were synthesized by PCR using linearized p18E7 plasmid as template, and PCR primers PCR4 and PCR5 (pM-900) and PCR2 and PCR3 (pM-270) (see Table **6.4**), then subcloned into the multiple cloning site (mcs) of the pUC18 plasmid (Table **6.1**). Promoter deletions pM-160, pM-350 and pM-470 were then derived from pM-900 by restriction enzyme digestion, and subcloned into the pUC18 mcs (Table **6.2**).

Of the reporter genes available at the time of these experiments, only the human growth hormone (hGH) gene has a secretable product, allowing for assay of promoter activity by measuring hGH protein in the cell media (Selden *et al*, 1986). A major advantage of this, apart from the ease of the assay, is that it allows measurement of the effects of particular cell treatments to be measured over a precise time period, in that immediately prior to the assay, all traces of hGH in the cell medium can be removed.

As the hGH gene cap site contains a fortuitous BamH1 site (DeNoto *et al*, 1981), this was used by Selden *et al* to subclone the hGH gene into the pUC12 plasmid vector, thus creating the p0GH vector (Selden *et al*, 1986). MIP1α promoter deletions pM-160, pM-350, pM-470 and pM-900, which each contain 36 bp of MIP1α 5'UTR sequences terminating in a 3' BamH1 site (Tables **6.2** and **6.4**), were cloned into p0GH with the p0GH BamH1 site at their 3' termini, and a HindIII site at their 5' termini, so that pGHM-160, pGHM-350, pGHM-470 and pGHM-900 contain 36 bp of MIP1α 5'UTR, followed directly by the complete hGH gene 5'UTR, followed by the hGH gene. The MIP1α promoter deletions pM-190 and pM-220 were subsequently synthesized by PCR (Tables **6.2** and **6.4**)and subcloned into p0GH in exactly this same way. The pM-270 MIP1α promoter deletion contains 46 bp of MIP1α 5'UTR sequences, terminating in a 3' Xba1 site (Tables **6.2** and **6.4**), so was cloned into p0GH with the p0GH Xba1 site (6 bp upstream from the p0GH BamH1 site), at its 3' terminus, and a HincII site at its 5' terminus. Thus, pGHM-270 contains 46 bp of MIP1α 5'UTR, followed by 6 bp of pUC12 sequences, followed by the complete hGH gene 5'UTR and the hGH gene. The integrity of pGHM constructs was confirmed by sequencing, using primer p0G1 (Table **6.3**), and a summary of all of the MIP1α promoter deletion/ hGH gene constructs made is shown in figure **10.1**.

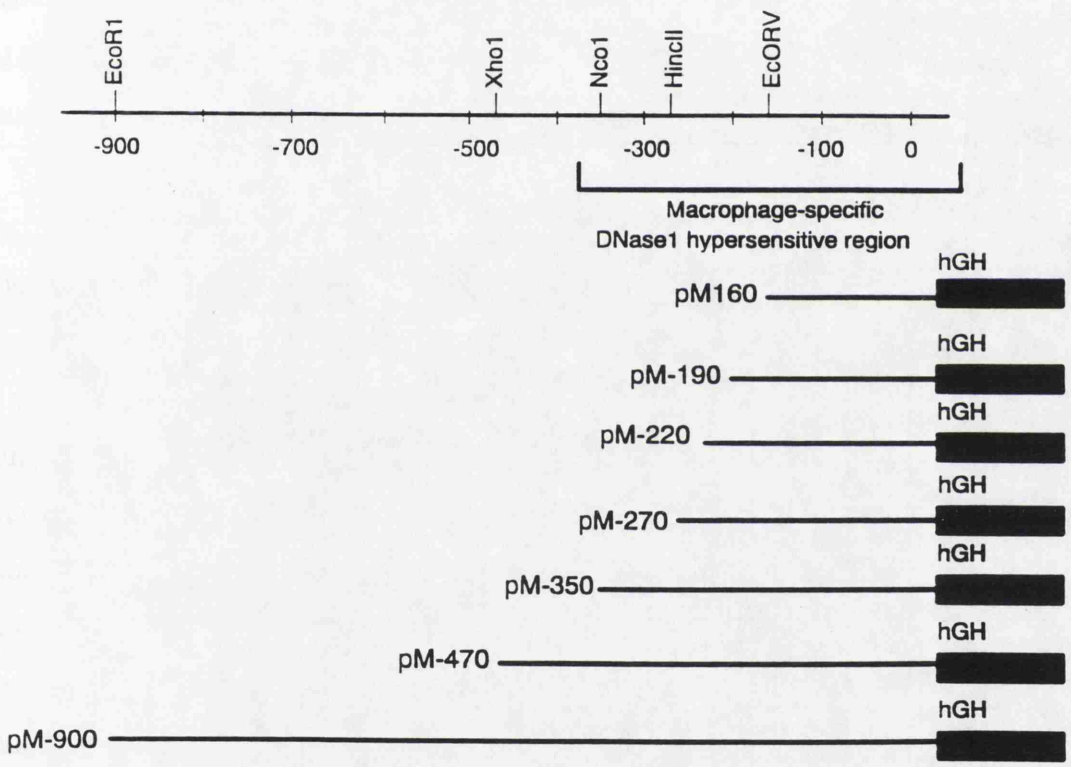
10.3 TRANSFECTION OF PLASMID DNA INTO MURINE CELLS.

10.3.1 Stable transfection versus transient transfection.

Test promoter/ reporter gene constructs transfected into mammalian cells can be assayed in stable- or transient transfection assays, both of which have several

Figure 10.1: Synthesis of MIP1α promoter deletion constructs.

Schematic representation of the MIP1α promoter deletion constructs synthesized using restriction enzyme digestion and PCR. At the top of the figure, the MIP1α promoter is shown, with distance (in bps) relative to the cap site (+1) and restriction enzyme recognition sites corresponding to the 5' ends of promoter constructs as indicated. The ~350 bp region of the promoter that is hypersensitive in macrophage cell-line nuclei is shown below the promoter sequence. Promoter sequences were cloned upstream of the human growth hormone (hGH) gene (filled boxes) in the promoterless p0GH plasmid. Promoter constructs pM-160, pM-190, pM-220, pM-350, pM-470 and pM-900 contain from +36 bps to -160 bp (EcoRV site), -190 bp, -220 bp, -270 bp (HincII site), -350 bp, -470 bp (Xho1 site) and -900 bp (EcoR1 site), respectively. Promoter construct pM-270 contains from +48 bp to -270 bp.



advantages and disadvantages. In stable transfections, the test DNA is co-transfected into the cells with $1/10^{th}$ of the number of copies of a plasmid encoding a selectable marker protein (I used the Homer 6 plasmid, which contains a gene encoding resistance to the antibiotic geneticin (G418)), so that any cells that incorporate the plasmid encoding the selectable marker into their genome are likely to also incorporate the test plasmid DNA. The G18-resistant cells are selected over a period of several weeks by incubating the transfected cells in cell growth medium containing G418, so that only cells that contain plasmid DNA stably-integrated into their genome survive.

The main disadvantage of stable transfectants is the length of time required to select then grow up sufficient antibiotic-resistant cells to allow the functional activity of test plasmids to be assayed. Another disadvantage is that, unless the stably-integrated test plasmid DNA contains LCR or boundary elements (see Introduction), the activity of the test promoter constructs is likely to be greatly affected by any gene regulatory elements (positive or negative) in the region of the site of integration. As different transfected cells contain plasmid DNA integrated into a different site in the genome, these position effects were minimized by pooling of >100 colonies derived from stably-transfected cells before assaying of test promoter activity. A final disadvantage of stable transfectants is that plasmid DNA incorporates into the genome of transfected cells in tandem repeats, rather than single copies, so that for comparison of the activity of different promoter constructs within and between cell-types, Southern blot analysis of the genomic DNA of transfected cells is required, in order to adjust the measured promoter activity for average copy number of stably-integrated test plasmid in each case.

An advantage of stable transfectants over transient transfectants (see below) is that repeated transfection of the cells is required in the latter case. Further, because stably-transfected cells are selected, virtually every cell in a stable transfection assay of promoter function contains the test plasmid, making the assay more sensitive than non-optimized transient transfections, in which only a few percent of the cells contain transfected DNA. Further, as the MIP1α gene is an early-response gene, its

expression is likely to be affected by the the short-term insult to the cells involved in the transfection procedures; for example, as Ca^{2+} is an integral part of several signal transduction pathways (see chapter **4**) exposure of the cells to high levels of Ca^{2+} in the calcium-phosphate co-precipitation method of transfection may affect the expression of the MIP1α gene in those cells. This problem is avoided in stably-transfected cells.

Transient transfection assays involve transfection of test plasmid DNA into cells, then assaying of test promoter activity within days, rather than weeks (see Methods section). Transfected plasmids are functionally assayed prior to integration into the genome, thus avoiding the problems this entails (see above). However, because the transfected plasmid DNA is unmethylated and not complexed with nucleosomes when introduced into the cells, this can occasionally lead to different (and spurious) results in transient transfections compared to stable transfections (see chapter **5**, section **5.3.3.3**, for a good example of this).

Unlike stable transfections, the efficiency of transfection must be optimized in transient transfection experiments, in order that sufficient plasmid copies are introduced into a sufficient proportion of the cells for the activity of transfected plasmid promoter constructs to be detectable. Furthermore, if too many copies of the plasmid are introduced into each transfected cell, spurious results might occur due to the excess ratio of test promoter constructs compared to available transcription factors; again, transient transfections must be optimized to avoid this problem.

Another problem with transient transfections is that a suitable reference plasmid, which does not interfere with the activity of the test plasmid, and which is co-transfected with the test plasmid to control for transfection efficiency, must be found (see below). Finally, in order to compare transient transfections of different cell types, a positive control promoter, whose activity is *defined* as being equivalent (but may perhaps not be in some cases) in all transfected cell types, must be used. In the experiments described here, I used the herpes simplex virus thymidine kinase (HSVTK) promoter linked to the hGH gene in the pHSVTKGH plasmid, as the positive control.

Beacause of the complex nature of the promoters of early-response genes (see chapter 3, section 3.3), I considered it likely that a large number of mutant MIP1α promoter constructs would be required in order to begin to understand the role of different promoter elements. Due to the length of time required to select and analyse a large number of stably-transfected promoter constructs, I analysed most wild-type and mutant MIP1α promoter deletion constructs in transient transfection assays, while initially confirming some of the transient transfection results obtained with pHSVTKGH, pGHM-160, pGHM-270 and pGHM-900 in stable transfection assays.

10.3.2 Reference plasmids encoding non-secreted reference proteins do not work in transient transfections of RAW 264.7 cells.

Two potential reference plasmids, pHSVβ-gal which contains the HSVTK promoter linked to the lacZ gene, encoding the β-galactosidase protein, and pSV-LUC which contains the SV40 promoter linked to the luciferase gene (see chapter 7 for description of assays for these proteins), were available in the laboratory at the initiation of this work. Neither the β-galactosidase nor the luciferase protein is secreted, unlike the hGH protein and the secreted alkaline phosphatase reference protein (see below).

In pilot experiments, the pHSVβ-gal and pSV-LUC plasmids were separately co-transfected with the MIP1α promoter pGHM-900 plasmid into either RAW 264.7-, MEL- or STO cells, and samples of cell media taken and cell-lysates made at 24 hour intervals after transfection. In all cases, the hGH protein was readily detectable in samples of cell media, but though both luciferase and β-galactosidase were readily detectable in MEL- and STO cell lysates, neither reference protein was detectable above background levels in assays of RAW 264.7 cell lysates (data not shown, as it is negative). Although the background in the β-galactosidase assay of RAW 264.7 cell lysates was high, possibly due to the presence of a similar endogenous protein (Chakraborty *et al*, 1989), the background in the luciferase assays was uniformly low. A possible reason for this was the presence of high levels of endogenous proteases in the RAW 264.7 cell lysates. I therefore included a range of protease inhibitors in the buffers used in the cell lysis and assay buffers, at equivalent concentrations to those

* (kindly donated by D.Wallace, Glaxo Group Research Ltd., Greenford, United Kingdom; Henthorn et al, 1988)

used in preparation of nuclear extracts (see chapter **7**). Inclusion of protease inhibitors did not affect detection of the luciferase protein in either MEL- or RAW 264.7 cell lysates. The possibility that the SV40 promoter does not work in RAW 264.7 cells can be discounted, as the pSV2ASPAP reference plasmid (see below) contains this promoter. The conclusion from this negative set of experiments was therefore that the non-secreted β-galactosidase and luciferase proteins were somehow being rapidly degraded in RAW 264.7 cells, while the hGH protein, chanelled into a secretory pathway, was in this way protected from destruction.

10.3.3 pSV2APAP, a plasmid encoding a secreted reference protein (SPAP) that is detectable in transient transfections of RAW 264.7 cells.

After the completion of the experiments described above, the pSV2ASPAP plasmid, containing the SV40 promoter linked to the gene encoding the secreted placental alkaline phosphatase (SPAP) reference protein, became available* in the laboratory. As predicted, this reference protein was readily-detectable (see below) in the cell growth medium of transiently transfected RAW 264.7 (as well as STO- and MEL) cells.

10.3.4 Methods of transfection: calcium phosphate-DNA co-precipitation and lipofection.

The first method I investigated for the transfection of plasmid DNA into murine cells was the widely-used calcium phosphate co-precipitation method (see chapter **7**), wherein a calcium phosphate-DNA precipitate is added to the cells, allowing the DNA to be efficiently taken up by the temporarily DNA-porous cell membranes. This method worked well, and was used to stably-transfect cells, but was not used in transient transfection assays for the reasons described in section **10.3.1.**

The lipofection method of mammalian cell transfection involves the coating of DNA in liposomes, which then fuse with the cell membrane, releasing the DNA into the cells. In the experiments described below, I used Lipofectin (GIBCO BRL), a commercially-available liposome preparation.

10.3.5 Optimization of parameters for Lipofectin-mediated transient transfections of plasmid DNA into RAW 264.7-, STO- and MEL cells.

The aim of the experiments described below was to obtain readily detectable levels of reporter- and reference proteins in the transient transfection of RAW 264.7, STO- and MEL cells, while remaining within the linear range of detection of the proteins.

As Lipofectin is variably toxic to different cell types, for each cell type, cells were plated as described (see chapter 7), then transfected with the appropriate amount of a Lipofectin-DNA mixture that contained 2 μg of pSV2ASPAP plasmid and either 0 μg, 5 μg, 10 μg, 30 μg or 50μg of Lipofectin reagent, and plates were incubated for 2 hrs, 5 hrs, 10 hrs or 15 hrs before replating of the Lipofectin-containing OPTIMEM medium with the appropriate volume of cell growth medium. Cells were examined at regular intervals throughout the experiment, and after 48 hours, cell media were tested for the presence of SPAP protein.

Examination of the cells showed that a large proportion of STO- and MEL cells were killed by incubation for 10-15 hours in the presence of Lipofectin, and that the 50 μg Lipofectin-DNA mixture was toxic to MEL cells. RAW 264.7 cells were maximally transfected by the 50 μg Lipofectin mixture for 15 hours, STO cells by the 50 μg Lipofectin mixture for 5 hours, and MEL cells by the 30 μg Lipofectin mixture for 5 hours, and the three cell types were henceforth routinely transfected using these conditions.

The pGHM-160, pGHM270 and pGHM-900 test plasmids and the pTKGH positive control plasmid were then included in the Lipofectin-DNA transfection mixture, and it was found that, with RAW 264.7 cells, 3 μg of test plasmid and 1.5 μg of reference plasmid gave adequate levels of detection, while with STO- and MEL cells, 8 μg of test plasmid and 1.5 μg of reference plasmid was required to obtain adequate levels of detection. These amounts of test- and control plasmids were termed the "standard" amounts of transfected DNA. In each case, no difference in test plasmid activity was detected when between 0.5 μg and 1.5 μg of reference plasmid was included in the Lipofectin-DNA mixture, demonstrating that 1.5 μg of reference plasmid had no effect on the levels of expression of test- and control plasmids. The amount of test plasmid was also varied, between 0.25-fold and 1.5-fold the standard

amounts of test plasmid DNA. As shown in figure **10.4** , the standard test plasmid DNA dilution used for transient transfection of RAW 274.7 cells is in the linear range. Similar data were obtained for STO- and MEL cell transfections, and the standard conditions outlined above were henceforth used in transient transfections of these cell types.

10.4 CELL-SPECIFIC BASAL ACTICITY OF MIP1α PROMOTER SEQUENCES.

The ability of serial deletions (figure **10.1**) of the MIP1α promoter to drive hGH reporter gene expression in RAW 264.7, MEL and STO cell-lines was examined using transient transfection assays. The activity of the promoter constructs in different cells was corrected for transfection efficiency by assaying secreted human placental alkaline phosphatase (SPAP) produced by co-transfection of the pSV2Apap reference plasmid, then compared to that of the positive control pHSVTKGH plasmid, which is assumed in experiments such as those described here to be equivalently expressed in each different cell-type used.

Endogenous MIP1α RNA levels are *basal* in RAW 264.7 cells after 72 hours' incubation in medium containing fresh 10% (v/v) FCS, and these cells are defined as being unstimulated (figures **9.1** and **9.2**). As the endogenous MIP1α mRNA levels in RAW 264.7 cells are rapidly and transiently induced by fresh 10% FCS (figures 8 and 9), this suggested that the serum-responsiveness of the endogenous MIP1α mRNA levels would be reflected in the transcriptional activity of MIP1α promoter sequences. Medium removed from unstimulated RAW 264.7 cells (defined as RAW 264.7 cell Conditioned Medium ("RAW CM", see chapter **7**)) was therefore used in transient transfection assays, in order to minimize the possible effects of fresh 10% (v/v) FCS on MIP1α promoter activity and thus measure the defined basal activity of MIP1α promoter constructs in RAW 264.7 cells. For comparison, the activity of MIP1α promoter constructs in transiently transfected MEL and STO cells was measured (figure **10.2**).

Figure 10.2: Ability of MIP1α promoter constructs to drive hGH gene expression in RAW 264.7 cells in RAW CM compared to that in STO and MEL cells.

Functional transient transfection studies of the activity of MIP1α promoter constructs linked to the hGH reporter gene. MIP1α promoter/hGH chimaeric constructs were co-transfected with reference plasmid pSV2APAP into RAW 264.7 (RAW), MEL or STO cells and then incubated for 36 hours in medium containing fresh 10% (v/v) FCS, after which SPAP gene expression was assayed in all transfectants and hGH gene expression was assayed in the MEL and STO transfectants. The RAW 264.7 transfectants were then PBS washed and incubated for a further 20 hour test period in RAW CM after which hGH gene expression was assayed. Assayed hGH gene expression was corrected for transfection efficiency (SPAP assay), and expressed relative to that of the pHSVTKGH positive control, which was given a value of 1 in STO and MEL cells and in RAW 264.7 cells in RAW CM. Values are the means of three experiments, in which each transfection was performed in triplicate. Assayed promoter constructs are shown to the left of the table.

Cell-type / Construct	MEL	STO	RAW
pM-160	0.18	0.07	5
pM-190	0.16	0.15	20
pM-220	0.14	0.2	21
pM-270	0.12	0.1	22
pM-350	0.1	0.14	19
pM-470	0.06	0.04	11
pM-900	0.08	0.04	15

As shown in figure **10.2**, in which all promoter activities are expressed as a multiple of pHSVTKGH activity (= 1 unit in all cell types), the maximal activity of any of the MIP1α promoter constructs in MEL or STO cells is 25-fold lower than the basal activity of the minimal MIP1α promoter construct pM-160 in RAW 264.7 cells. The basal activity in RAW 264.7 cells of each of constructs pM-190, pM-220, pM-270 and pM-350 is a further 4-fold greater than that of pM-160 in RAW 264.7 cells (figure **10.2**). Hence, the maximal basal activity of the MIP1α promoter in RAW 264.7 cells is approximately 100-fold greater than the maximal unstimulated activity of the MIP1α promoter in MEL or STO cells (figure **10.2**). This is consistent with the presence of basal levels of MIP1α mRNA in unstimulated RAW 264.7 cells but not in STO- or MEL cells (figure **9.2**), and the presence of a strong MIP1α promoter DNase1 hypersensitive site in macrophage cell lines which is not detected in STO- or MEL cells (figures **9.3** and **9.4** and negative data not shown). RAW 264.7 cells stably transfected with the pM-160, pM-270 and pM-900 promoters were also compared to that of the pHSVTKGH construct in stably transfected RAW 264.7 cells, and the relative activities of pM-270 and pHSVTKGH were found to be comparable to those obtained in transient transfection assays using the same constructs. However, whereas in the transient transfection assays, pM-900 had lower activity than pM-270 (figure **10.2**), these two promoters were equally active in stable transfection assays (figure **10.6**). This is an apparently common phenomenon in transient transfection assays, where longer promoter constructs containing redundant 5' sequences have lower activity than shorter constructs containing the same functional sequences. This correlates with the fact that only the proximal ~350 bp of the MIP1α promoter is DNase1 hypersensitive in macrophage cell-lines (figures **9.3** and **9.4**). The 5-fold lower relative activity of the pM-160 promoter in stable- compared to transient transfection assays can be attributed to the fact that the transfection efficiency was very low in the transfection used to generate the pGHM-160 stable-transfectants, suggesting that the plasmid cop-number in this stably-transfected cell-line is low compared to that in the pHSVTKGH-, pGHM-270- and pGHM-900 stable-transfectants.

10.5 MIP1α PROMOTER ACTIVITY IS STIMULATED BY LPS AND FRESH SERUM.

As MIP1α mRNA rapidly accumulates in RAW 264.7 cells in response to LPS or serum stimulation (figures **9.1**, **9.2** and **10.8a**), I tested whether this is reflected at the level of transcription. Transiently-transfected unstimulated RAW 264.7 cells were PBS-washed twice, then incubated for the defined 20-hour test period (see chapter **7**) in either RAW CM, RAW CM containing 2 μg/ml LPS, fresh medium containing fresh 10% (v/v) FCS, or fresh medium containing fresh 10% (v/v) FCS and 2 μg/ml LPS.

All the promoter constructs are on aggregate 2.7- to 3.8-fold more active in fresh 10% (v/v) FCS than in RAW CM over the 20 hour test period (figure **10.3**). Maximal activity in fresh 10% (v/v) FCS was obtained with the pM-220 construct, although maximal serum stimulation was obtained with the pM-160 construct (figure **10.3**). Both the basal and stimulated activities of transiently-transfected promoter constructs pM-470 and pM-900 in RAW 264.7 cells is lower than that of the pM-190 to pM-350 constructs (figure **10.3**). This apparently common phenomenon of transient transfections was discussed in section **10.4**.

Although the promoter of the human MIP1α gene LD78α contains a putative serum response element (SRE) (Widmer et al, 1991), and the MIP1α promoter contains a putative Related to Serum Response Factor (RSRF) binding site, which has been shown to confer serum-responsiveness on other promoters (CTATTTCTAG: Widmer *et al*, 1991; Han *et al*, 1992), these sites are further upstream from the proximal promoter region shown to be serum responsive in this study (figure **10.3**). It could therefore be argued that the "serum response" observed is a result of transfection of an excessive number of copies of plasmid constructs into the cells, which contain limiting numbers of the transcription factors that bind to the MIP1α promoter. In this scenario, addition of fresh 10% FCS to the cells would stimulate rapid cell proliferation and division, in which each daughter cell would theoretically receive half the parental number of copies of test plasmid, hence effectively doubling

Figure 10.3: Effects of fresh 10% (v/v) FCS and LPS on the expression of chimaeric MIP1α promoter/ hGH gene constructs in RAW 264.7 cells.

Functional transient transfection studies of the activities of MIP1α promoter/ hGH gene constructs in RAW 264.7 cells: effects of RAW CM (CM) or fresh 10% (v/v) FCS in the presence or absence of 2 μg/ml of LPS. Constructs were co-transfected with reference plasmid pSV2APAP into RAW 264.7 cells and then incubated for 36 hours in medium containing fresh 10% (v/v) FCS, after which SPAP gene expression was assayed. Transfected cells were then PBS washed *in-situ*, and incubated for a further 20 hour test period in either RAW CM (CM), RAW CM plus 2 μg/ml of LPS (CM + LPS), new medium containing fresh 10% (v/v) FCS (10% FCS), or new medium containing fresh 10% (v/v) FCS and 2 μg/ml LPS (10% FCS + LPS), after which hGH gene expression was assayed. Assayed hGH gene expression was corrected for transfection efficiency (SPAP assay), and expressed relative to that of the pHSVTKGH positive control, which was given a value of 1 in RAW 264.7 cells in RAW CM. For each construct, fold-induction of expression obtained by the various treatments is expressed in parenthesis relative to the level of expression of the construct in RAW CM. Values are the means of three experiments, in which each transfection was performed in triplicate. Assayed promoter construct are shown to the left of the table.

	basal	induced		
Treatment	CM	CM + LPS	10% FCS	10% FCS + LPS
Construct				
pM-160	5	14 (x 2.8)	19 (x 3.8)	29 (x 5.8)
pM-190	20	31 (x 1.6)	57 (x 2.9)	77 (x 3.9)
pM-220	21	24 (x 1.1)	66 (x 3.1)	55 (x 2.6)
pM-270	22	30 (x 1.4)	65 (x 3.0)	73 (x 3.3)
pM-350	19	25 (x 1.3)	53 (x 2.8)	49 (x 2.6)
pM-470	11	14 (x1.3)	35 (x3.2)	34(x3.1)
pM-900	15	18 (x1.2)	40(x2.7)	38(x2.5)

the number of transcription factors available per transfected copy of the MIP1α promoter. The result of this would be a "serum response" of the cells treated with fresh 10% FCS compared to the less rapidly proliferating cells treated with RAW CM over the same test period. In order to test this idea, transient transfections were undertaken using a range of dilutions of the MIP1α promoter/ hGH gene plasmid DNA. The serum-responsiveness in RAW 264.7 cells of both MIP1α promoter constructs pM-160 and pM-270 is approximately equivalent over this range (figure **10.4**), thus suggesting that the serum-responsiveness of these promoters in RAW 264.7 cells is not artefactual.

The MIP1α promoter is responsive to LPS, and the LPS responsive element appears to be located within the pM-160 construct, as the LPS-responsiveness is effectively lost as the promoter length is increased beyond 190 bp (pM-190) (figure **10.3**). In order to further investigate this, the LPS responsiveness of the pM-160 promoter was investigated over a range of concentrations of fresh serum. The greatest effect (4.5-fold) of LPS on the activity of this promoter was observed in the presence of fresh 1% FCS, compared to 2.8-fold in RAW CM, 2.1-fold in the presence of fresh 5% FCS and 1.5-fold in the presence of fresh 10% FCS (figure **10.5**). The effect on pM-160 promoter activity of LPS and 1% FCS, LPS and 5% FCS and (slightly less so) LPS and 10% FCS, is approximately additive (figure **10.5**).

Investigation of the LPS-responsiveness in RAW CM, of stably-transfected pHSVTK-, pM-160 and pM-900 promoters confirms that the pM-160 promoter contains an LPS response element (figure **10.6**). The stably-transfected promoter activities were tested over an 11-hour timecourse, while the transiently-transfected promoter activities were tested over a 20-hour period; this suggests that the greater LPS-responsiveness of the pM-160 promoter in stably-transfected- compared to transiently-transfected is because the LPS up-regulation of transcription mediated by the pM-160 promoter is transient.

In summary, the functional data presented suggests that the MIP1α promoter contains strong basal elements between +36 bp and -160 bp, and between -160 bp and -220bp, plus LPS- and serum-responsive elements between +36 bp and -160 bp. As

Figure 10.4: Test for whether the observed "serum responsiveness" of MIP1α promoter/hGH gene chimaeric constructs is due to excessive copy number of transiently transfected plasmids.

Functional transient transfection studies of the activity of MIP1α promoter/hGH constructs in RAW 264.7 cells: effects of plasmid copy number on basal- and serum-induced expression. Defining the standard 0.3 μg of chimaeric MIP1α promoter/hGH gene plasmid DNA per plate used in transient transfections as "1 standard dilution unit", 0.25-, 0.5-, 0.75- and 1 dilution units of this test DNA per plate (made up to 0.3 μg with pUC18 carrier DNA) was then used in separate transfection assays. Constructs were co-transfected with pSV2APAP reference plasmid into RAW 264.7 cells and then incubated for 36 hours in medium containing fresh 10% (v/v) FCS, after which SPAP gene expression was assayed. Transfected cells were then PBS washed *in-situ* and incubated for a further 20 hour test period in either RAW CM (CM) or new medium containing fresh 10% (v/v) FCS (10% FCS), after which hGH gene expression was assayed. Assayed hGH gene expression was corrected for transfection efficiency (SPAP assay), and expressed relative to that of the pHSVTKGH positive control, which was given a value of 1 in RAW 264.7 cells in RAW CM. For each dilution of a construct, fold-induction of expression obtained by the fresh 10% (v/v) FCS treatment ("induced") is expressed in parentheses relative to the level of expression of the same dilution of that construct in RAW CM ("basal"). Values are the means of two experiments, in which each transfection was performed in triplicate. Assayed promoter constructs are shown to the left of the table.

	basal				induced			
Treatment	CM				10% FCS			
DNA dilution (x standard)	0.25	0.5	0.75	1.0	0.25	0.5	0.75	1.0
Construct								
pM-160	1.2	1.9	2.5	5	3.7 (x 3.1)	7.3 (x 3.8)	9.2 (x 3.7)	15.7 (x 3.1)
pM-270	4.5	10.8	13.2	22.8	10.7 (x 2.4)	25.0 (x 2.3)	34.8 (x 2.6)	42.7 (x 1.9)

Figure 10.5: Investigation of the LPS-responsiveness of MIP1α promoter/hGH gene chimaeric construct pM-160.

Functional transient transfection studies of the activity of MIP1α promoter/hGH gene chimaeric construct pM-160 in RAW 264.7 cells: effects of different concentrations of fresh serum on the LPS responsiveness of the pM-160 construct. Construct pM-160 was co-transfected with reference plasmid pSV2APAP into RAW 264.7 cells and then incubated for 36 hours in medium containing fresh 10% (v/v) FCS, after which SPAP gene expression was assayed. The transfected cells were then PBS washed *in-situ*, and incubated for a further 20 hour test period in either RAW CM (CM), new medium plus fresh 1% (v/v) FCS (1%FCS), new medium plus fresh 5% (v/v) FCS (5% FCS) or new medium containing fresh 10% (v/v) FCS (10% FCS), in the presence (+) or absence (-) of 2 μg/ml LPS, after which hGH gene expression was assayed. Assayed hGH gene expression was corrected for transfection efficiency (SPAP assay), and expressed relative to that of the pHSVTKGH positive control, which was given a value of 1 in RAW 264.7 cells in RAW CM. Fold-induction of expression by the various treatments, over the level of expression of pM-160 in RAW CM, is shown in parentheses. Values are the means of two experiments, in which each transfection was performed in triplicate.

Treatment	CM		1% FCS		5% FCS		10% FCS	
LPS	-	+	-	+	-	+	-	+
Relative Expression	5	14 (x 2.8)	7.5	34 (x 4.5)	18	38 (x 2.1)	19	29 (x 1.5)

Figure 10.6: Expression of chimaeric MIP1α promoter/ hGH gene constructs in stably-transfected RAW 264.7 cells.

Functional stable transfection studies of the activities of MIP1α promoter/ hGH gene constructs in RAW 264.7 cells: effects of RAW CM in the absence (- LPS) or presence (+LPS) of 2 μg/ml of LPS. Stably-transfected RAW 264.7 cell cultures, containing cells derived from >100 pooled colonies, were grown for 3 days in medium containing 900 μg/ml of geneticin and 10% (v/v) fresh FCS. Cells were then PBS washed *in-situ*, before incubation for 11 hours in RAW CM or RAW CM containing 2 μg/ml of LPS, after which hGH gene expression was assayed. Assayed hGH gene expression was expressed relative to that of the pHSVTKGH positive control, which was given a value of 1 in RAW 264.7 cells in RAW CM. For each construct, fold-induction of expression obtained by the various treatments is expressed in parenthesis relative to the level of expression of the construct in RAW CM. Values are the mean of 2 experiments, in which each transfection was performed in triplicate. Assayed promoter constructs are shown to the left of the table.

	-LPS	+LPS
pHSVTK	1	1 (1)
pM-160	1	6.5 (6.5)
pM270	21	29 (1.4)
pM-900	19	27 (1.4)

the endogenous MIP1α mRNA levels are induced only transiently by fresh serum or LPS in RAW 264.7 cells (figure **9.2**), this suggests that the stimulation of transcription in response to these agents might also be transient, and this is supported by the stable-transfectant data. However, as the transfection studies were performed over an 11- (stable) or 20-hour (transient) period, it is not possible to define the precise magnitude and kinetics of the induction of transcription; nuclear run-on analysis (see chapter **15**) would be required to investigate this.

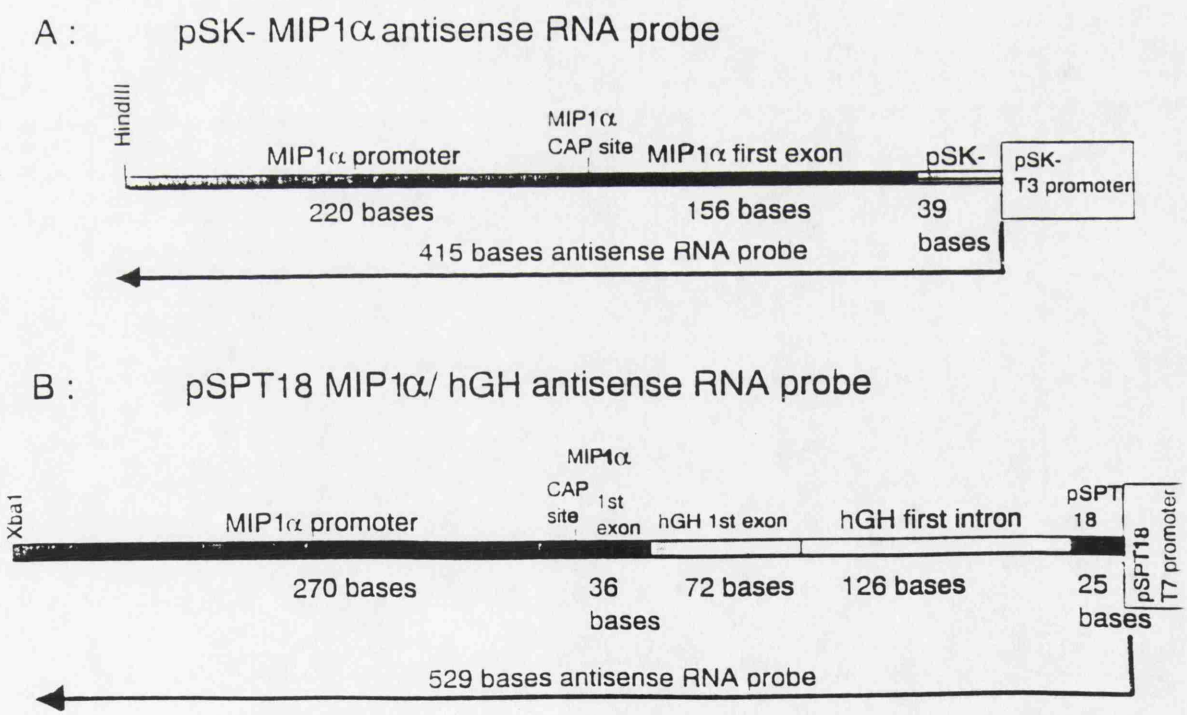
10.6 CORRECT TRANSCRIPTIONAL INITIATION OF MIP1α PROMOTER CONSTRUCTS.

To demonstrate that hGH gene expression observed with the MIP1α promoter/hGH constructs represented transcriptional initiation at the MIP1α gene cap site, I first confirmed that transcription of the endogenous MIP1α gene initiates at the proposed MIP1α gene CAP site in RAW 264.7 cells, then demonstrated that transcription in the above chimaeric constructs initiates at the same site. In order to examine these points, total RNA was prepared from both untransfected RAW 264.7 cells and RAW 264.7 cells stably transfected with construct pM-900, and analysed using the RNase protection assay with the probes described below. The reason that stably-, rather than transiently-transfected RAW 264.7 cells were used in this assay was partially because I reasoned that more of the chimaeric RNA would be present in total RNA from the stably-transfected cells, and partially a matter of not wasting expensive transfection reagents on transfection of the large number of RAW 264.7 cells required to provide an adequate RNA sample.

Endogenous MIP1α mRNA levels in RAW 264.7 cells were initially examined in the RNase protection assay using the pSK-MIP1α plasmid (figure **10.7a** and Tables **6.1** and **6.4**), which is designed to detect correctly-initiated MIP1α mRNA. When this antisense RNA probe was used in RNase protection analysis of total RNA from RAW 264.7 cells, an ~156 base RNA fragment was protected (figure **10.8a**), corresponding to correctly-initiated MIP1α mRNA encoded by the MIP1α first exon (see figure **10.7a**).

Figure 10.7: Antisense RNA probes for the detection of correctly-initiated endogenous MIP1α gene RNA and transfected chimaeric MIP1α/hGH gene RNA.

Antisense RNA probes used in RNase protection analysis to detect the cap site used in the endogenous MIP1α gene and to confirm that the same cap site is used by the chimaeric MIP1α/hGH gene. **A:** pSK-MIP1α antisense RNA probe construct was synthesized by PCR using plasmid p18S2 as template, and cloned into the multiple cloning site (*mcs*) of pSK⁻ plasmid. MIP1α promoter, first exon and cap site are as shown. The orientation of the cloned MIP1α sequences in the pSK⁻vector is such that cutting with restriction enzyme HindIII in the flanking pSK⁻ *mcs* sequences (as shown) allows a 415 base pSK-MIP1α antisense RNA transcript (including 39 bases of pSK⁻ sequences) initiating at the pSK⁻ T3 RNA polymerase promoter to be synthesized *in-vitro*. **B:** pSPT18-MIP1α/hGH antisense RNA probe construct was generated by HincII/BalI restriction enzyme digestion of pGHM-350 plasmid, and cloned into the *mcs* of pSPT18 plasmid. MIP1α cap site, promoter and first exon sequences, and hGH first exon and first intron sequences are as shown. The orientation of the cloned sequences in the pSPT18 vector is such that cutting with XbaI restriction enzyme in the pSPT18 *mcs* sequences (as shown), allows a 529 base antisense RNA transcript (including 25 bases of pSPT18 plasmid sequences) initiating at the pSPT18 T7 RNA polymerase promoter to be synthesized *in-vitro*.



To demonstrate that MIP1α/ hGH gene chimaeric constructs initiate transcription at the same site as the endogenous gene in RAW 264.7 cells, plasmid pSPT18MIP1α/hGH was constructed (Table **6.1** and figure **10.7b**) and used to produce uniformly [^{32}P-α]-UTP-labelled 529 base chimaeric MIP1α/ hGH antisense RNA transcripts, which were incubated with total RNA from either untransfected (control) or pGHM-900 stably-transfected RAW 264.7 cells and analyzed by the RNase protection assay. With control RNA, no protection was observed (therefore not shown), but with the latter RNA, an ~108 base RNA fragment is protected, corresponding to a chimaeric mRNA that comprises 36 bases encoded by the MIP1α first exon and 72 bases encoded by the hGH first exon, and which is thus correctly initiated at the MIP1α gene cap site (see figure **10.7b** for diagram, and figure **10.8b** for resulting RNase protection). Because the pSPT18MIP1α/hGH plasmid contains 126 bp of hGH first intron-encoding sequences (figure **10.7b**), and total RNA, rather than polyA$^+$ mRNA, was used in these experiments, it is likely that the ~230 base protected RNA observed in figure **10.8b** is an unspliced chimaeric MIP1α /hGH pre-mRNA fragment of 234 bases.

RAW 264.7 cell serum stimulation results in transient accumulation of MIP1α mRNA, initiated from the MIP1α gene cap site (figure **10.7a**). Probe pSPT18MIP1α/hGH was used in a similar RNase protection experiment, to test whether chimaeric MIP1a/hGH RNA is also transiently induced by serum in RAW 264.7 cells, so reflecting transient upregulation of MIP1α promoter activity in response to serum. Although the endogenous MIP1α mRNA contains 3' UTR instability sequences (see chapter **4**, section **4.6**) which are not present in the 3' UTR of hGH mRNA (DeNoto *et al*, 1981), this experiment was carried out in light of similar experiments with regions of the TNFα cytokine gene linked to the hGH gene (Han *et al*, 1991), which showed that in the absence of actinomycin D, the cytokine 3' UTR sequences made no difference to the kinetics of transient LPS-induced RNA accumulation driven by the TNFα promoter. However, results from the MIP1α experiment were inconclusive (figure **10.8c**), as the chimaeric RNA appears to be transiently induced over the first 3 hours of serum stimulation, then gradually

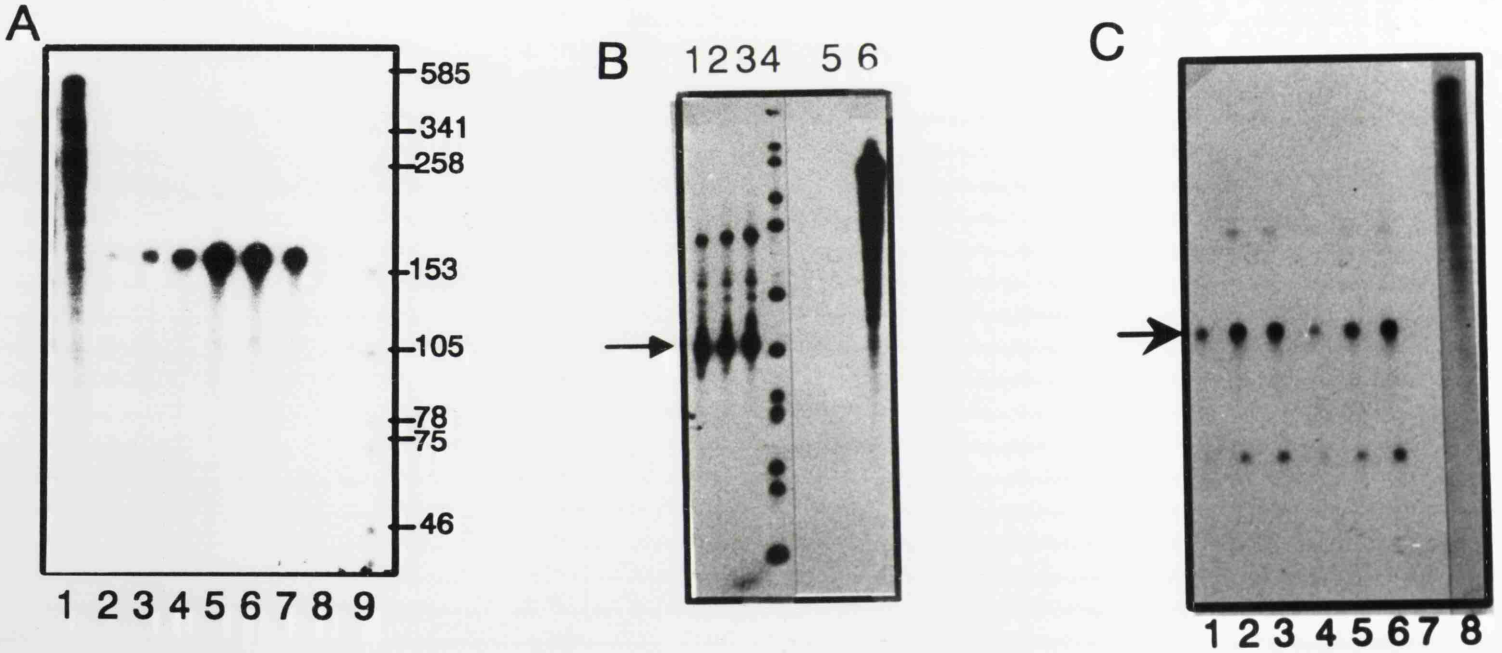

A
585
341
258
153
105
78
75
46
1 2 3 4 5 6 7 8 9
B
1234 5 6
C
1 2 3 4 5 6 7 8

Figure 10.8: Detection of correctly-initiated endogenous MIP1a RNA and chimaeric MIP1α/hGH gene RNA in RAW 264.7 cells by RNase protection analysis.

RNase protection analysis of total RNA prepared from RAW 264.7 cells stably transfected with the MIP1α gene promoter pM-900/hGH gene construct. **A:** Cells were incubated for 3 days after replating in SLM containing fresh 10% (v/v) FCS, then treated as follows before extraction of total RNA: Lane 2, no treatment control; lanes 3-7, incubation in medium containing fresh 10% (v/v) FCS for 30 mins, 90 mins, 3 hrs, 6 hrs and 9 hrs respectively. 20 μg of total RNA or 20 μg of control yeast RNA was then hybridized for 24 hours at 45°C in an RNase protection assay (RPA) with a uniformly [α-^{32}P]-UTP-labelled 415 base antisense MIP1α RNA riboprobe (synthesized from linearized pSK-MIP1α plasmid template). Hybrids were then digested with an RNaseA/RNaseT1 mixture (5 μg/ml and 100 U/ml respectively) and protected RNA fragments resolved by denaturing PAGE and autoradiography. Lanes 1 and 8, yeast control RNA/ riboprobe mixture: mock-digested (lane 1) or RNase-digested (lane 8); lane 9, molecular weight markers: plasmid pUC18, restriction-digested with Sau3AI and 5' end-labelled using [γ-^{32}P]-ATP and polynucleotide kinase). The size (in bases) of the molecular weight markers is shown. The correctly-initiated MIP1α RNA protected is ~156 bases long, as seen on the autoradiograph. **B:** Total RNA was prepared from unstimulated RAW 264.7 cell pM-900 stable-transfectants. 20 μg aliquots of total RNA (lanes 1-3) or 20 μg of control yeast RNA (lanes 5 and 6) were then hybridized for 24 hours at 45°C in an RPA with the uniformly [α-^{32}P]-UTP labelled 529-base antisense MIP1α/hGH RNA riboprobe (synthesized from linearized pSPT18-MIP1α/hGH plasmid template). Hybrids were then digested with an RNaseA/T1 mixture, as follows: Lane 1, 10 μg/ml and 200 U/ml, respectively; lane 2, 5 μg/ml and 100 U/ml; lanes 3 and 5, 0.5 μg/ml and 10 U/ml. Lane 6, mock-digested probe/ yeast RNA mixture. Protected RNA fragments were resolved by denaturing PAGE and autoradiography. Lane 4, molecular weight markers, as for (**A**). The probe protects a 103 base corrrectly-initiated MIP1α/hGH chimaeric gene RNA, which is denoted by the arrow to the left of the autoradiograph. A slower-migrating 230 base protected RNA is probably correctly initiated unspliced MIP1α/hGH chimaeric RNA, as discussed in the text. **C:** 20 μg aliquots of the RNA stocks used in (**A**) were used in RNase protection experiments with the riboprobe used in (**B**). RNase digestions of all RNA samples were as used in (**A**). The RNA samples, from the serum stimulation experiment as described in (**A**), were: Lane 1, control; lane 2, 30 mins, lane 3, 90 mins, lane 4, 3 hrs, lane 5, 6 hrs, lane 6, 9 hrs. Lanes 7 and 8, RNase-digested and mock-digested yeast RNA/ riboprobe mixtures, respectively. The 103 base correctly-initiated protected MIP1α/hGH chimaeric RNA is identified by the arrowhead to the left of the autoradiograph. The faster-migrating fainter bands may be due to the probe used in this experiment being particularly degraded (see lane 8).

accumulate again. Although such biphasic induction of mRNA accumulation is not without precedent (Schwartz,B.S. *et al*, 1992), this experiment was not repeated as the probe used is close to the lower size limit recommended for use in RNase protection assays, so that quantitation of protected RNA may not be reliable, and adequate control probes were not available.

CHAPTER 11: INVESTIGATION OF TRANSCRIPTION FACTOR BINDING *IN-VITRO* TO THE MIP1α PROXIMAL PROMOTER.

11.1 OVERVIEW.

The described DNase1 hypersensitivity studies (figures **9.3** and **9.4**) suggest that, within an ~10 kbp locus containing the MIP1α gene, only the ~350 bp proximal promoter region is functionally important. Functional transfection studies with MIP1α promoter constructs (figures **10.2-10.5**) suggested that in RAW 264.7 cells, the proximal promoter region (+36 bp to -220 bp) is sufficient to confer a high basal level of macrophage-specific transcription, and serum- and LPS-responsiveness, on a linked reporter gene. This information suggested that transcription factor(s) conferring macrophage-specific transcription and LPS- and serum-inducibility upon a reporter gene, bind to the MIP1α gene proximal promoter.

To investigate such transcription factor binding, I used two *in-vitro* assays: *in-vitro* DNase1 footprinting (footprinting) and electrophoretic mobility shift assays (EMSAs). In both of these assays, double stranded DNA probes are 5' end-labelled using polynucleotide kinase and [^{32}P-γ]-ATP: footprinting probes, which are typically several hundred bps in length, are labelled on one strand only, while EMSA probes are typically 20-40 bp in length and are labelled on both strands (chapter **7**). In both assays, the purified 5' end-labelled probes are incubated with crude nuclear extracts from the required cell-type, in the presence of a large excess of non-specific competitor DNA, poly(dI.dC).poly(dI.dC). Transcription factors bind specifically and

with high affinity to short recognition sequences on DNA, but can also bind weakly to random DNA sequencesin a non-specific manner. The presence of a large excess of poly(dI.dC).poly(dI.dC) in these assays competes for non-specific binding, while allowing specific high-affinity binding of particular transcription factor(s) to the labelled probe sequences. Small amounts of non-specifically DNA binding proteins, for example that don't bind well to poly(dI.dC).poly(dI.dC), can be distinguished from specific transcription factor binding by the inclusion in the assays of either an excess of unlabelled probe sequence (~25-fold) or a larger excess (eg. 100 to 200-fold) of an unrelated sequence (that may nevertheless be known to bind specifically to certain different transcription factor(s)); if DNA binding is specific, the latter should not compete for the binding while the former should compete for most if not all of the binding.

The footprinting assay involves limited DNase1 digestion of the probe-nuclear extract solution, so that any sequences specifically bound by transcription factor(s) will be preferentially protected from DNase1 digestion, and these sequences are detected as gaps (footprints) in the DNase1-digested probe ladder upon visualization by denaturing PAGE and autoradiography. An advantage of this assay is that, by Maxam-Gilbert sequencing (Sambrook *et al*, 1989) of the labelled probe, and resolving the sequence in tandem with the footprint reactions, it is possible to determine the precise *in-vitro* transcription factor binding sites over a large region of DNA. Furthermore, if, for example, transcription factors bound co-operatively to two different regions of the footprinted sequence, the footprinting assay (in contrast, generally, to the EMSA assay) would allow such binding to be observed. Problems with the footprinting assay are: a) its low sensitivity, in that a particularly low abundance transcription factor might bind to only 10%, for example, of the labelled probe (ie. only a very weak, at best, footprint would be observed); b) its limited resolution, in that, for example, if three different transcription factors are capable of binding to exactly the same sequences in a particular DNA site, this would be very difficult to distinguish using the footprinting assay; c) in a DNA-bound transcription

factor complex containing several proteins, only the components of that complex that are in contact with the DNA are detectable in the footprinting assay.

In EMSAs, the DNA probe-protein complexes formed in the described incubation period are resolved by low ionic strength non-denaturing PAGE. A major advantage of this assay is that low-abundance transcription factors are easily detected, as this assay is capable of detecting binding to only a small percentage of the total probe. Further, due to the large excess of labelled probe, all transcription factor complexes that can bind to a particular probe sequence are able to form, and can be electrophoretically resolved, as different nucleoprotein-DNA complexes generally migrate differently in EMSAs, according to their size, charge and conformation. Finally, the affinity of binding of specific nucleoprotein complexes to a particular DNA sequence is easily examined in EMSAs, by titration of the -fold molar excess of unlabelled specific competitor oligonucleotides incubated with the labelled probe/ crude nuclear extract/ poly(dI.dC).poly(dI.dC) mixture.

11.2 *IN-VITRO* DNase1 FOOTPRINT PROTECTION ASSAYS.

The p18M-350 plasmid (Table **6.1**) contains the pM-350 promoter fragment cloned into the mcs of the pUC18 plasmid, such that the plasmid can be restriction enzyme digested at either the 5' or 3' end (with respect to the direction of the MIP1α gene), 5' end-labelled, then restriction enzyme digested at the opposite end of the fragment, to release the footprinting probe, 5' end-labelled on the required DNA strand.

Probes generated in the above manner were incubated with crude large-scale nuclear protein extracts (see chapter **7**) from RAW 264.7 cells (unstimulated or 12 hour LPS-stimulated) and control STO and MEL cells. In each of these cell-types, six sequences, termed FP-a to FP-f, within the proximal ~220 bp of the MIP1α promoter are protected in these assays, on the coding strand of the DNA (figure **11.1**). Site FP-b is A+T-rich and therefore inefficiently cleaved by DNase1, but binding to FP-b was deduced due to enhanced cleavage of sequences flanking the A+T-rich region (figures **11.1a** and **11.1b**). No sequences further 5' to this region up to ~ -220 bp are

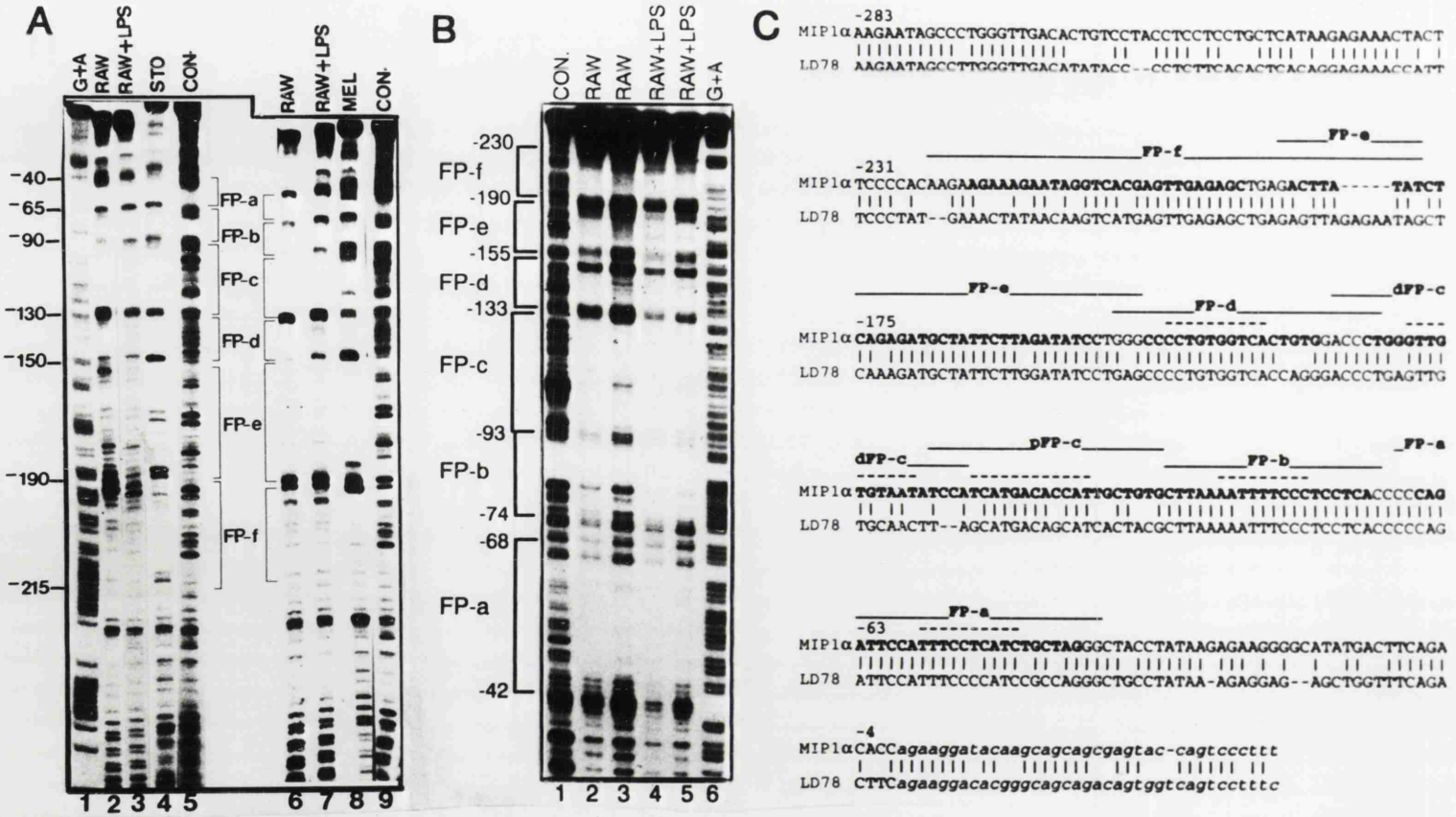

A
G+A
RAW
RAW+LPS
STO
CON-
RAW
RAW+LPS
MEL
CON-
-40
-65
-90
-130
-150
-190
-215
FP-a
FP-b
FP-c
FP-d
FP-e
FP-f
1 2 3 4 5 6 7 8 9
B
CON.
RAW
RAW
RAW+LPS
RAW+LPS
G+A
-230
FP-f
-190
FP-e
-155
FP-d
-133
FP-c
-93
FP-b
-74
-68
FP-a
-42
1 2 3 4 5 6
C
-283
MIP1α AAGAATAGCCCTGGGTTGACACTGTCCTACCTCCTCCTGCTCATAAGAGAAACTACT
LD78 AAGAATAGCCTTGGGTTGACATATACC--CCTCTTCACACTCACAGGAGAAACCATT
FP-e
FP-f
-231
MIP1α TCCCCACAAGAAGAAAGAATAGGTCACGAGTTGAGAGCTGAGACTTA-----TATCT
LD78 TCCCTAT--GAAACTATAACAAGTCATGAGTTGAGAGCTGAGAGTTAGAGAATAGCT
FP-e
dFP-c
FP-d
-175
MIP1α CAGAGATGCTATTCTTAGATATCCTGGGCCCCTGTGGTCACTGTGGACCCTGGGTTG
LD78 CAAAGATGCTATTCTTGGATATCCTGAGCCCCTGTGGTCACCAGGGACCCTGAGTTG
dFP-c
pFP-c
FP-b
FP-a
MIP1α TGTAATATCCATCATGACACCATTGCTGTGCTTAAAATTTTCCCTCCTCACCCCCAG
LD78 TGCAACTT--AGCATGACAGCATCACTACGCTTAAAAATTTCCCTCCTCACCCCCAG
FP-a
-63
MIP1α ATTCCATTTCCTCATCTGCTAGGGCTACCTATAAGAGAAGGGGCATATGACTTCAGA
LD78 ATTCCATTTCCCCATCCGCCAGGGCTGCCTATAA-AGAGGAG--AGCTGGTTTCAGA
-4
MIP1α CACCagaaggatacaagcagcagcgagtac-cagtcccttt
LD78 CTTCagaaggacacgggcagcagacagtggtcagtcctttc

Figure 11.1: Investigation of nucleoprotein binding to the MIP1α promoter using *in-vitro* DNaseI footprinting.

In-vitro DNaseI footprint protection assay of the MIP1α promoter, using promoter fragment pM-350 as probe. Plasmid p18M-350, in which the MIP1α promoter pM-350 is cloned into the HincII/BamH1 sites of the *mcs* of pUC18, was digested with either HincII (**A**) or EcoR1 (**B**), 5'end-labelled using [α-^{32}P]-ATP and polynucleotide kinase, so labelling either the coding strand (**A**) or the non-coding strand (**B**), then digested with EcoR1 (**A**) or HincII (**B**), to release the probe, which was gel-purified. 5' end-labelled probe (1 ng) was incubated in the absence (CON) or presence of crude nuclear extracts prepared from MEL, STO and unstimulated- (RAW) or 13 hr LPS-stimulated (RAW + LPS) RAW 264.7 cells. After limited DNaseI digestion (CON, 800 ng/ml; nuclear extract-containing, 8 μg/ml), nucleic acids were resolved by denaturing polyacrylamide gel electrophoresis. G+A: sequence of the pM-350 MIP1α promoter (**A**, coding sequence; **B**, non-coding sequence), prepared by the G+A chemical sequencing reaction of the labelled probe. The six protected regions, and their positions (in bps) relative the MIP1α gene cap site, are shown. **A:** Probe was incubated with 0 μl (CON, lane 5), 60 μl (lanes 2 to 4 and 8), or 80 μl (lanes 6 and 7) of nuclear extract. **B:** Probe was incubated with 0 μl (CON, lane 1), 80 μl (lanes 2 and 4) or 60 μl (lanes 3 and 5) of nuclear extract. **C:** sequence of the MIP1α proximal promoter. Sequences protected on the coding strand of the MIP1α promoter (**A**) in the footprint experiments (FP-a to FP-f) are in bold typeface, sequences used to generate double-stranded oligonucleotides for EMSA studies (FP-a to FP-f) are shown as solid lines, and core protein recognition motifs are shown as dashed lines. The sequence of the human MIP1α (LD78α) gene proximal promoter (Nakao *et al*, 1990) is aligned beneath the MIP1α sequence, with vertical lines showing sequence identity.

protected (figure **11.1** and data not shown (as there is nothing to see)). Each of the described footprints, FP-a to FP-f, are also protected on the non-coding strand using unstimulated- or 12 hour LPS-stimulated RAW 264.7 cell nuclear extracts (figure **11.1b**). In all cases save FP-a, each footprint extends slightly further distally on the non-coding strand compared to the coding strand (figures **11.1a** and **11.1b**), reflecting a more extended region of transcription factor binding to the non-coding strand compared to the coding strand of the MIP1α promoter at these sites.

Figure **11.1c** shows a summary of the footprinting data. Footprints FP-a to FP-f are marked, and the sequences corresponding to the coding-strand footprint in each case are marked in bold typeface (figure **11.1c**). In conclusion, the footprint protection pattern observed with the different cell nuclear extracts revealed several small differences. These differences may be due to slightly different concentration of nuclear proteins used in each case, and to slightly different conditions in the footprinting reactions, for example in terms of reducing conditions (large-scale nuclear protein preparation involves an overnight dialyzation step, and the DTT in the solutions is likely to become inactive over such a long period). Nevertheless, from the above data, it appears that the three cell-types examined contain nuclear protein(s) which are capable of binding to five of the identified protein binding sites (all except FP-b, see below) *in-vitro*.

In order to examine nuclear protein binding to these sites in greater detail, double stranded oligonucleotides encompassing sites FP-a to FP-f were synthesized as probes for nuclear protein binding in EMSAs. Site FP-c contains two potential transcription factor binding sites (proximal and distal), and hence two overlapping olignucleotides (pFP-c and dFP-c, respectively) were used to investigate this site. These oligonucleotides (oligos) are shown as bold lines above each of the footprinted sequences FP-a to FP-f shown in figure **11.1c**, and were designed to encompass both the coding- and non-coding strand footprinted sequences identified in the experiments shown in figures **11.1a** and **11.1b**.

11.3 PRELIMINARY IDENTIFICATION OF C/EBP FAMILY MEMBERS BINDING TO THE PROXIMAL MIP1α GENE PROMOTER, AND DELINEATION OF THE DNA SITE TO WHICH THEY BIND.

11.3.1 Multiple C/EBP transcription factor family binding sites in the proximal MIP1α promoter.

MIP1α promoter footprinted sites FP-a, pFP-c, dFP-c and FP-d contain sequences similar to those shown previously to bind members of the C/EBP family of proteins. The proposed sites for binding of C/EBP family members to these footprinted sequences are shown as dotted lines above the footprinted sites marked in figure **11.1c**, and a sequence comparison of these proposed MIP1α promoter C/EBP family binding sites and known C/EBP family binding sites is shown in figure **11.2a**.

The C/EBP family of proteins are notoriously able to specifically bind to a wide variety of DNA sequences, with superficially little sequence homology between different sites (Akira *et al*, 1990; Landschultz *et al*, 1988; Lee *et al*, 1993); however, certain core sequences are conserved between the known- and proposed C/EBP family binding sites (figure **11.2a**).

Nuclear protein binding to the MIP1α promoter footprinted sequences FP-a, pFP-c, dFP-c and FP-d was therefore compared with that to three characterized sites: a) the murine G-CSF gene promoter element 1 (mGPE-1), as mGPE-1 is an LPS response element in macrophages and has been shown to bind C/EBP family members *in vitro* (Akira *et al*, 1990; Nishizawa *et al*, 1990); b) the related sequence from the human G-CSF promoter, hG-CSF (Shannon *et al*, 1990); c) a documented C/EBP family binding sequence, originally shown to bind C/EBPα (Landschultz *et al*, 1988).

EMSA analysis of labelled FP-a probe with nuclear extracts from unstimulated RAW 264.7 cells revealed five nucleoprotein complexes, a1 to a5 (figure **11.2b**). Complexes a1, a2 and a3 were specifically competed out by excess FP-a, pFP-c, dFP-c, FP-d, mGPE-1, hG-CSF and C/EBP competitors (figure **11.2b**).Complex a4 will be discussed separately below, and complex a5 is variably detected and competed so its significance is unclear (see below and Discussion). As shown in figure **11.2c**,

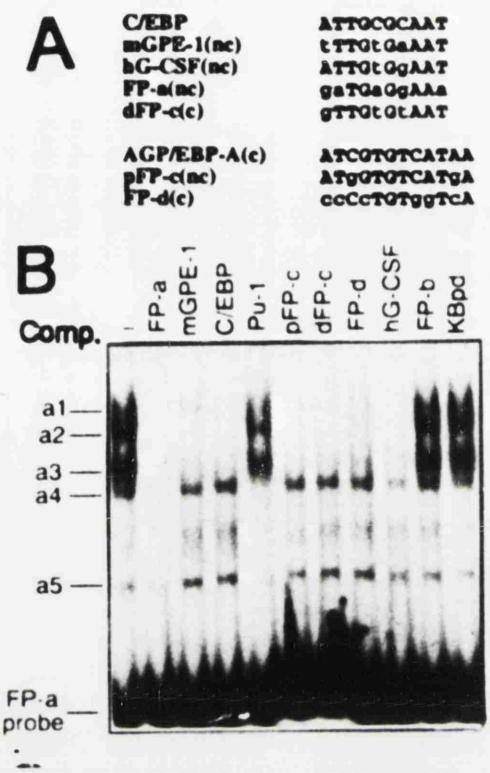

A
C/EBP ATTGCGCAAT
mGPE-1(nc) tTTGtGaAAT
hG-CSF(nc) ATTGtGgAAT
FP-a(nc) gaTGaGgAAa
dFP-c(c) gTTGtGtAAT
AGP/EBP-A(c) ATCGTGTCATAA
pFP-c(nc) ATgGTGTCATgA
FP-d(c) ccCcTGTggTcA
B
Comp.
-
FP-a
mGPE-1
C/EBP
Pu-1
pFP-c
dFP-c
FP-d
hG-CSF
FP-b
KBpd
a1
a2
a3
a4
a5
FP-a probe
C
-
FP-a
C/EBP
dFP-1
hG-CSF
Pu-1
aP3a
a1
a2
a3
a4
free probe

Figure 11.2: EMSA detection of C/EBP family- containing nucleoprotein complexes binding to the proximal MIP1α promoter.

EMSA anslysis of RAW 264.7 cell nuclear protein binding to MIP1α proximal promoter footprinted site FP-a. **A:** Sequence comparison of MIP1α promoter sites FP-a, pFP-c, dFP-c and FP-d with known C/EBP family binding sites. Top of figure: upper case letters denote identity- and lower case non-identity, respectively, with corresponding nucleotides in the C/EBP family binding site of the C/EBP oligonucleotide (Landschultz *et al*, 1988). Bottom of figure: identity- and non-identity with nucleotides in the AGP/EBP-A C/EBP family binding site (Lee *et al*, 1993) are denoted as above. (c), coding strand; (nc), non-coding strand. **B:** EMSAs in which 0.5μg of nuclear extract from unstimulated RAW 264.7 cells was incubated with 1 ng of FP-a probe in the absence (-) or presence (+) of 25 ng (FP-a, mGPE-1, C/EBP, PU.1) or 100 ng (κBpd, pFP-c, dFP-c, FP-d, hG-CSF, FP-b) of competitor (Comp.). C/EBP-related retarded complexes a1-a3, the PU.1/SpiB-related complex a4 and the variably detected a5 complex are labelled. The identity of a variably-detected complex visible between a4 and a5 is unknown, and the complex is unlabelled. Excess uncomplexed FP-a probe (bottom of the autoradiograph) is labelled. **C:** EMSAs in which 0.5 μg of nuclear extract from 8 hr LPS-stimulated (2 μg/ml) RAW 264.7 cells was incubated with FP-a probe in the absence (-) or presence (+) of 25 ng (FP-a, C/EBP, dFP-c, hG-CSF or PU.1) or 100 ng (αp3a), of competitor (Comp.). C/EBP-related retarded complexes a1-a3, PU.1/Spi-B-related complex a4 and uncomplexed excess FP-a probe (free probe) are labelled. Variably-detected complex a5 is not visible.
Coding-strand sequences of oligonucleotides:
FP-a: CCCAGATTCCATTTCCTCATCTGCTAGGG
pFP-c: TCCATCATGACACCATTGCTGTG
dFP-c: ACCCTGGGTTGTGTAATATCCA
FP-d: GGGCCCCTGTGGTCACTGTGGACCCT
C/EBP: GCTGCAGATTGCGCAATCTGCAGC (Landschultz *et al*, 1988)
hG-CSF: GGATCAGAGATTCCACAATTTC (Shannon *et al*, 1990)
mGPE-1: ACAGAGATTCCCCGATTTCACAAAAACTTTCGC (Nishizawa *et al*, 1990)
PU.1: ATAACCTCTGAAAGAGGAACTTGGTTAGGT (Klemsz *et al*, 1990)
κBpd: CAACGGCAGGGGAATTCCCCTCTCCTT (Ballard *et al*, 1990)
FP-b: CTTAAAATTTTCCCTCCTCAC
αp3a: GATCCAAACCAGCCCAATGAGAACTGCTCCA (MacLeod *et al*, 1991)

incubation of FP-a probe with nuclear extracts from LPS-stimulated RAW 264.7 cells revealed the same five nucleoprotein complexes, a1 to a5. Again, complexes a1 to a3 were specifically competed by excess FP-a, C/EBP, dFP-c or hG-CSF oligos, but not by an unrelated competitor αp3a (MacLeod *et al*, 1991) (figure **11.2c**).

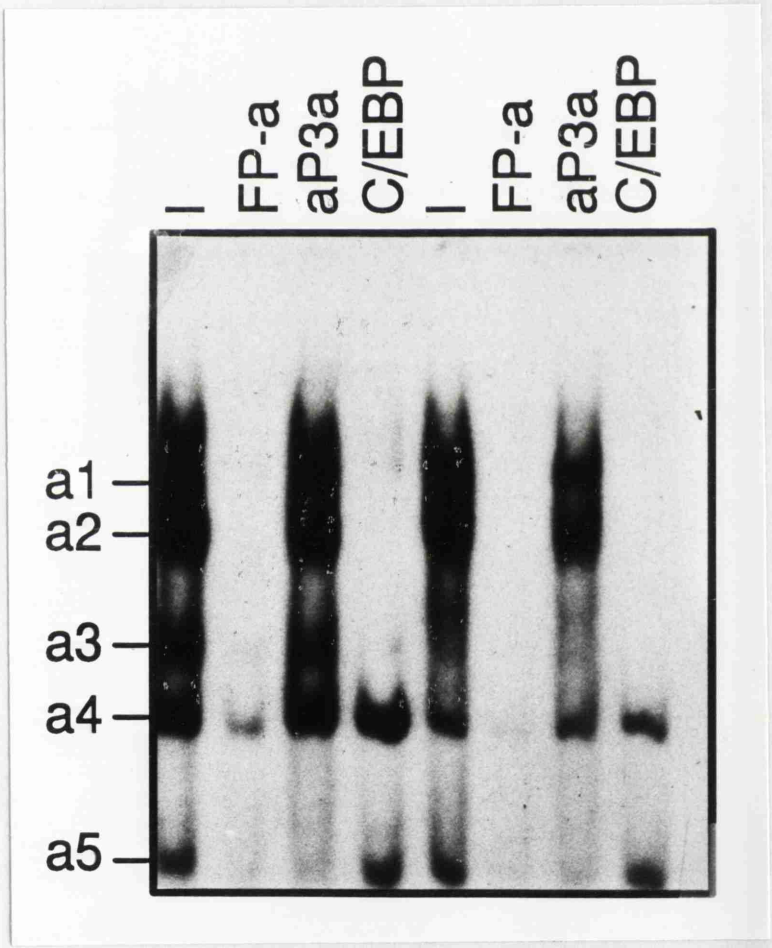
When nuclear extracts from RAW 264.7 cells incubated for 24 hours with medium containing either fresh 10% FCS or LPS are incubated with FP-a probe, a similar pattern of nuclear protein binding is obtained, in that bands a1 to a5 are formed with both extracts (figure **11.3**) and, as for unstimulated RAW 264.7 cell nuclear extracts, bands a1 to a3 are again specifically competed by FP-a or C/EBP oligos, but not by non-specific competitor αp3a (figure **11.3**). In the experiments shown in figure **11.3**, ~1 μg of nuclear extract was used in lanes 1-4, compared to ~0.5 μg in lanes 5-8. In light of this information, it appears that very similar levels of complexes a1, a2, a4 and a5 are formed whether RAW 264.7 cells have been stimulated for 24 hours with fresh 10% FCS or 2 μg/ml of LPS (figure **11.3**), though a new band migrating slightly slower than band a3 is observed in the latter case (figure **11.3**). In both cases, complexes a1 and a2 are more intense than complex a3, which is markedly different to the results obtained with the same probe and nuclear extracts from unstimulated RAW 264.7 cells (see below).

Similar studies using labelled pFP-c, dFP-c, FP-d, C/EBP and mGPE-1 probes and nuclear proteins from control or LPS stimulated RAW 264.7 cells revealed three nucleoprotein complexes which were consistently present in each case, namely complexes 1, 2 and 3, where complexes 1 and 2 co-migrate with complexes a1 and a2 respectively formed with FP-a probe (figure **11.4a**). It was shown in figure **11.2** that these probes used as cold competitors specifically compete out complexes a1 to a3 formed with labelled FP-a probe. As shown in figure **11.4b**, the reverse is also true, in that excess FP-a competitor specifically competes out complexes 1 to 3 formed with probes pFP-c, dFP-c, FP-d and C/EBP, while κBpd competitor does not compete for these complexes.

FP-a competes poorly for complex 1 formed with pFP-c probe (figure **11.4b**), with a 100-fold excess FP-a required for significant competition. However, a 200-fold

Figure 11.3: Effects of fresh 10% (v/v) FCS or LPS on nucleoprotein-DNA complex formation in EMSAs with MIP1α promoter site FP-a.

EMSA anslysis of RAW 264.7 cell nucleoprotein binding to MIP1α proximal promoter site FP-a. 1 μg of nuclear extract from RAW 264.7 cells that had been incubated for 24 hours in SLM containing fresh 10% (v/v) FCS (lanes 1-4) or in RAW CM in the presence of 2 μg/ml LPS (lanes 5-8), was incubated in EMSAs with 1 ng of probe FP-a in the absence (-) or presence of 25 ng (FP-a, C/EBP) or 100 ng (αp3a) of competitor (Comp.). The C/EBP-related (a1, a2 and a3) and PU.1/Spi-B-related (a4) retarded nucleoprotein complexes (see text) are shown, as is the variably-detected a5 nucleoprotein complex.
Coding-strand sequences of oligonucleotides:
FP-a: CCCAGATTCCATTTCCTCATCTGCTAGGG
C/EBP: GCTGCAGATTGCGCAATCTGCAGC (Landschultz *et al*, 1988)
αP3a: GATCCAAACCAGCCAATGAGAACTGCTCCA (MacLeod *et al*, 1991)



excess of κBpd competitor does not compete at all for complex 1 (or any other complex) formed with this probe or probes dFP-c, FP-d or C/EBP (figure **11.4b**). Further, as shown in figure **11.4b**, a 25-fold excess of pFP-c competitor efficiently competes for complex a1 formed with FP-a probe. Hence, it can be concluded that the same protein(s) form complex a1 and complex 1, but that site pFP-c binds this complex with significantly higher affinity than does site FP-a.

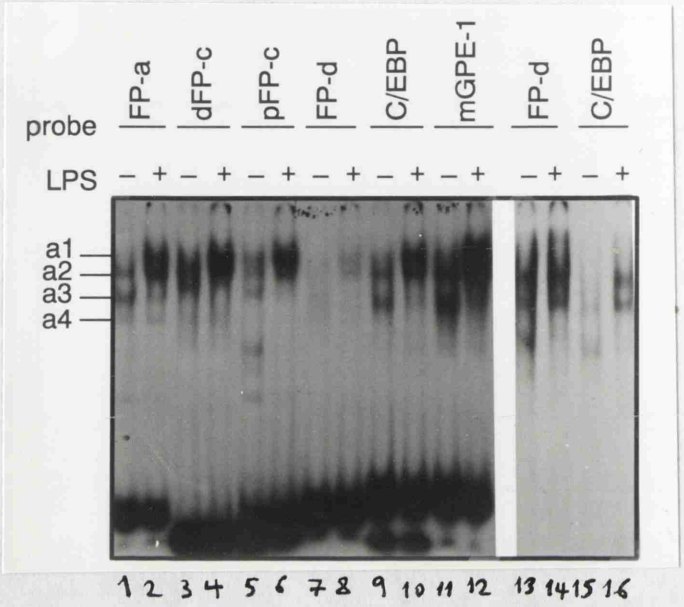
When binding to FP-a, dFP-c, pFP-c, FP-d, C/EBP and mGPE-1 probes by control and LPS stimulated (5 hours) RAW 264.7 nuclear extracts was compared (figure **11.4a**), a consistent pattern emerged: the intensity of binding in complexes a1 and a2 (with FP-a probe) and complexes 1 and 2 (the other probes) increase compared to a3 or complex 3 after RAW 264.7 LPS stimulation. As complexes 1 and 2 obtained with dFP-c, pFP-c, FP-d, C/EBP and mGPE-1 probes and complexes a1 and a2 obtained with FP-a probe co-migrate, cross-compete and exhibit a similar response to LPS stimulation of RAW 264.7 cells (figures **11.2-11.4**), this suggests they are equivalent nucleoprotein complexes.

11.3.2 UV-crosslinking studies of C/EBP family-containing nucleoprotein-DNA complexes.

11.3.2.1 Overview.

UV crosslinking of nucleoproteins to DNA is a method of determining the approximate molecular size of those proteins. In this technique, the double-stranded EMSA oligonucleotide to which the protein complex of interest binds in EMSAs is resynthesized, but with bromodeoxyuridine (BrdU) substituted for chosen thymidine nucleotides, and the resynthesized oligonucleotide is 5' end-labelled and used in EMSAs. BrdU can be then be covalently crosslinked to any protein that is touching it, by exposing the protein-DNA complex to ultraviolet light for a short time, so that the more BrdU residues within the region of contact of protein and DNA, the greater the chance of crosslinking. Once the protein-DNA complexes binding to the BrdU-substituted probe have been resolved in an EMSA, the wet gel is then exposed to UV light, the covalently-crosslinked protein-DNA complexes of interest excised, the protein denatured, and the radiolabelled protein-DNA complex resolved by SDS-

A


probe
FP-a
dFP-c
pFP-c
FP-d
C/EBP
mGPE-1
FP-d
C/EBP
LPS
– + – + – + – + – + – + – + – +
a1
a2
a3
a4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

B

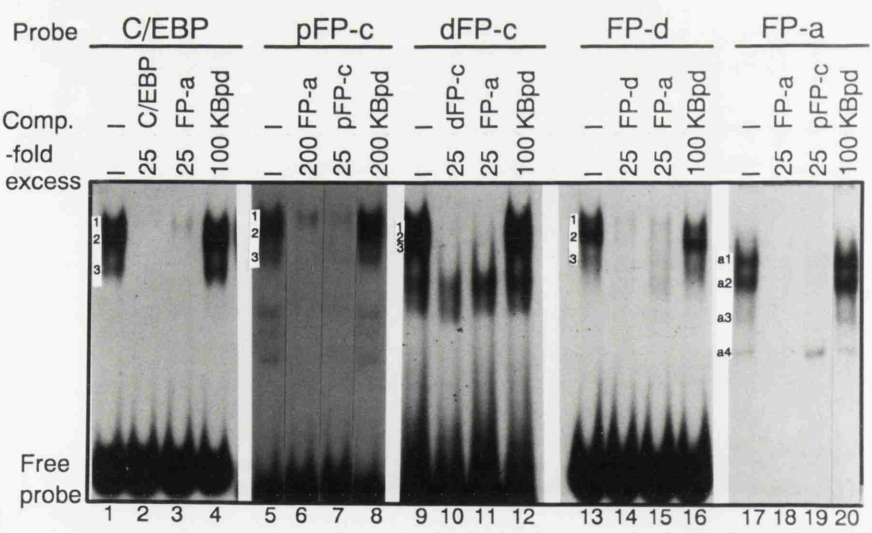

Probe
C/EBP
pFP-c
dFP-c
FP-d
FP-a
Comp.
-fold
excess
– C/EBP 25 FP-a 25 100 KBpd
– 200 FP-a 25 pFP-c 200 KBpd
– 25 dFP-c 25 FP-a 100 KBpd
– 25 FP-d 25 FP-a 100 KBpd
– 25 FP-a 25 pFP-c 100 KBpd
1
2
3
a1
a2
a3
a4
Free
probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 11.4: Similar C/EBP family nucleoprotein binding to MIP1α proximal promoter sites in EMSAs.

EMSA anslysis of RAW 264.7 cell nuclear protein binding to MIP1α promoter sites FP-a, pFP-c, dFP-c and FP-d, and to known C/EBP family binding sites C/EBP and mGPE-1. **A:** 1 ng of FP-a, pFP-c, dFP-c, FP-d, C/EBP or mGPE-1 probe was incubated in EMSAs with either 0.5 μg (lanes 1-12) or 2.5 μg (lanes 13-16) of nuclear extract prepared from either unstimulated (LPS -) or 5 hr LPS-stimulated (LPS +) RAW 264.7 cells. C/EBP-related retarded nucleoprotein complexes a1 to a3 and PU.1/SpiB-related complex a4 (FP-a probe) are marked. Further faint faster-migrating C/EBP-related complexes are also formed with pFP-c probe and are discussed in the text. Faster-migrating complexes formed with dFP-c probe are non-specific. Excess probe (free probe) migrates as indicated. **B:** 1 ng of C/EBP, pFP-c, dFP-c, FP-d or FP-a probe was incubated in EMSAs with nuclear extracts from 4 hr LPS-stimulated RAW 264.7 cells in the absence (-) or presence of the -fold molar excess of competitor (Comp.) shown. C/EBP-related nucleoprotein complexes a1 to a3 and PU.1/SpiB nucleoprotein complex a4 (FP-a probe) and C/EBP-related complexes 1 to 3 (other probes) are marked. Faster-migrating C/EBP-related complexes formed with pFP-c probe and non-specific complexes formed with dFP-c probe are as in (A). Uncomplexed excess probe migrates as shown (free probe).
Coding-strand sequences of oligonucleotides:
FP-a: CCCAGATTCCATTTCCTCATCTGCTAGGG
pFP-c: TCCATCATGACACCATTGCTGTG
dFP-c: ACCCTGGGTTGTGTAATATCCA
FP-d: GGGCCCCTGTGGTCACTGTGGACCCT
mGPE-1: ACAGAGATTCCCCGATTTCACAAAAACTTTCGC (Nisizawa *et al*, 1990)
C/EBP: GCTGCAGATTGCGCAATCTGCAGC (Landschultz *et al*, 1988)
PU.1: ATAACCTCTGAAAGAGGAACTTGGTTAGGT (Klemsz et al, 1990)
κBpd: CAACGGCAGGGGAATTCCCCTCTCCTT (Ballard *et al*, 1990)

PAGE and autoradiography. In SDS-PAGE, the denatured protein is coated in negative charge, so that generally all covalently-linked protein-DNA complexes are resolved on the basis of molecular size. A limitation of this assay is that only the DNA-binding component of any nucleoprotein complex formed with a particular probe can be covalently crosslinked to the DNA.

11.3.2.2 UV-crosslinking studies of LPS stimulated C/EBP-related complexes a1 and a2 formed with FP-a probe.

In order to further investigate the nucleoprotein binding to site FP-a, the double stranded FP-a probe was resynthesised to incorporate bromodeoxyuridine (BrdU) residues in place of thymidine residues within the proposed site of binding of C/EBP family proteins (figure **11.5a**). Within the coding strand of FP-a, six BrdU residues were incorporated, five of which are within the proposed C/EBP family binding site (figure **11.5a**). Due to the nature of the FP-a sequence, only two BrdU residues were incorporated into the non-coding strand, only one of which lies within the proposed C/EBP family binding site (figure **11.5a**).

The BrdU-containing FP-a probe was [γ-^{32}P]-ATP-labelled and used as a probe in an EMSA assay with unstimulated or LPS stimulated RAW 264.7 nuclear extracts, as per unmodified FP-a probe. Several controls were included in this experiment: in parallel to the above, the unmodified FP-a probe was used in the EMSA with the same nuclear extracts, in the presence or absence of FP-a, PU.1 or κBpd (non-specific) competitors, to ensure that the correct specific complexes were excised in the experiment with BrdU-substituted FP-a; a second reaction using the BrdU-substituted probe was also carried out, and the samples treated identically to the first BrdU-containing probe samples, except that these were not exposed to UV light. These second samples were resolved by SDS-PAGE adjacent to the relevant UV-crosslinked samples.

The molecular size of the modified FP-a probe is approximately 18 kDa (figure **11.5b**) so that to obtain the estimated molecular size of nucleoproteins crosslinked to this probe, 18 kDa should be deducted from the apparent size of the protein-DNA complexes resolved by SDS-PAGE. Hence, complex a4 contains two crosslinked

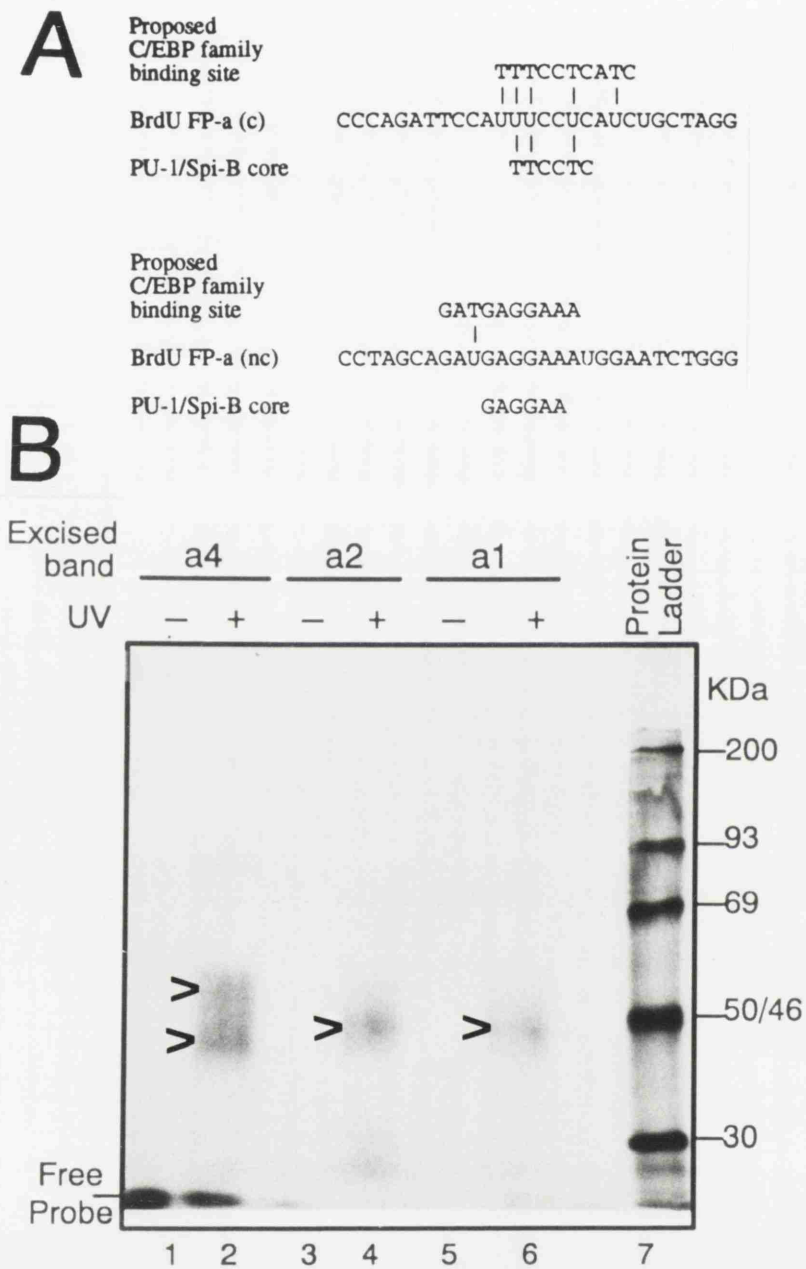

A
Proposed C/EBP family binding site
TTTCCTCATC
BrdU FP-a (c)
CCCAGATTCCAUUUCCUCAUCUGCTAGG
PU-1/Spi-B core
TTCCTC
Proposed C/EBP family binding site
GATGAGGAAA
BrdU FP-a (nc)
CCTAGCAGAUGAGGAAAUGGAATCTGGG
PU-1/Spi-B core
GAGGAA
B
Excised band
a4
a2
a1
UV
– + – + – +
Protein Ladder
KDa
200
93
69
50/46
30
Free Probe
1 2 3 4 5 6 7

Figure 11.5: Molecular size measurements of nucleoproteins binding to MIP1α proximal promoter site FP-a, using UV-crosslinking.

UV crosslinking of the a4 and LPS-stimulated a1 and a2 RAW 264.7 cell nucleoprotein complexes to probe FP-a. **A:** The coding (c)- and non-coding (nc) strands of the bromodeoxyuridine (BrdU)-substituted double-stranded FP-a oligonucleotide used in UV-crosslinking studies are shown, with "U" denoting BrdU nucleotides. The proposed C/EBP-family- and PU.1/SpiB protein core binding sites of the unsubstituted FP-a probe are aligned, and vertical lines denote corresponding thymidine and BrdU nucleotides in the unsubstituted- and BrdU-substituted sequences, respectively. **B:** The BrdU-substituted FP-a probe was used in EMSAs with 10 μg of nuclear extract from unstimulated- (lanes 1 and 2) or five-hour LPS-stimulated (lanes 3-6) RAW 264.7 cells. Duplicates of each of complexes a1, a2 and a4 were either not exposed (-) or exposed (+) to UV irradiation, and resolved by SDS PAGE next to protein molecular weight markers ("protein ladder"). The molecular size of the protein markers is shown to the right of the autoradiograph, and the non-UV-crosslinked BrdU-substituted FP-a probe migrated as shown (free probe). Specific crosslinked proteins present in complexes a1, a2 and a4 are marked by arrowheads.

proteins of approximate molecular sizes 37 (=55-18) kDa and 30 (=48-18) kDa, while complexes a1 and a2 both contain one crosslinked protein of approximate size 32 (=50-18) kDa (figure **11.5b**).

Proteins present in complex a4 are of different molecular sizes to those forming complexes a1 and a2 (figure **11.5b**). This is as expected, as FP-a probe complexes a1 and a2 are competed by C/EBP family binding competitors, while complex a4 is not (figures **11.2-11.4**). Further, in terms of molecular size, crosslinked protein components of complexes a1 and a2 are similar if not identical to each other, and are of similar size (after subtraction of 18 kDa for the FP-a probe) to the two C/EBP family proteins that are known to be expressed in macrophages, namely C/EBPβ and C/EBPδ, which have MWts of 31KDa and 29KDa respectively (Scott *et al*, 1992). In contrast, C/EBPα has a molecular size of ~42 kDa (Scott *et al*, 1992), while the molecular size of the C/EBPγ protein is not known).

Hence, complexes 1/a1 and 2/a2 formed in EMSAs with MIP1α promoter sites FP-a, pFP-c, dFP-c and FP-d cross-compete, co-migrate and respond similarly to LPS stimulation of RAW 264.7 cells (figures **11.2-11.4**), and a DNA binding component of complexes a1 and a2 has the same molecular size as C/EBP family proteins that are present in macrophages (figure **11.5b**). This suggests that complexes 1/a1 and 2/a2 contain C/EBPβ and/or C/EBPδ.

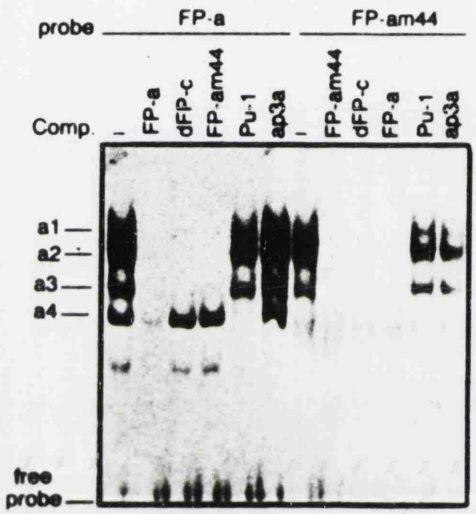
11.3.3 C/EBP-related complexes a3 formed with FP-a probe and 3 formed with probes C/EBP, mGPE-1, hG-CSF, pFP-c, dFP-c and FP-d are equivalent.

Complexes 3 and a3 obtained with these probes cross-compete and have a similar response to LPS treatment of RAW 264.7 cells, but do not always co-migrate in EMSAs (figures **11.2-11.4**). The C/EBP family binding site in FP-a was identified by sequence homology (figure **11.2a**) and UV-crosslinking studies (figure **11.5**). In order to further confirm the FP-a C/EBP family binding site, 2 bp changes, which mutate the proposed C/EBP family binding site, were introduced into FP-a, giving FP-am41 (figure **11.6a**), and the FP-am41 probe was used in EMSAs. EMSA studies, using RAW 264.7 cell nuclear extracts, show that C/EBP-related complexes a1, a2 and a3

A


C/EBP
atTGcGcAAt
FP-am44(nc)
CCTAGCAGtTGtGtAAtTGGAATCTGGG
FP-a(nc)
CCTAGCAGATGAGGAAATGGAATCTGGG
FP-am41(nc)
CCTAGCAaAaGAGGAAATGGAATCTGGG
PU-1
aAaGAGGAAc

B


probe
FP-a
FP-am44
Comp.
–
FP-a
dFP-c
FP-am44
Pu-1
ap3a
–
FP-am44
dFP-c
FP-a
Pu-1
ap3a
a1
a2
a3
a4
free probe

C

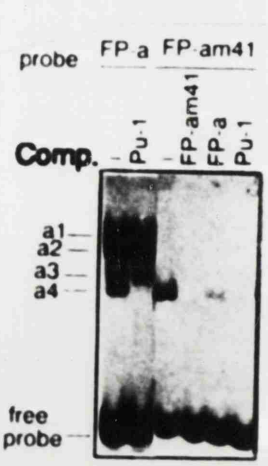

probe
FP-a
FP-am41
Comp.
–
Pu-1
–
FP-am41
FP-a
Pu-1
a1
a2
a3
a4
free probe

Figure 11.6: Investigation of complex 3/a3 formed with MIP1α promoter C/EBP family binding site probes in EMSAs.

EMSA anslysis of RAW 264.7 cell nuclear protein binding to wild-type and mutant forms of MIP1α promoter site FP-a. **A:** Non-coding (nc) strands of mutant FP-a sequences FP-am44 and FP-am41 are aligned with the non-coding strand of the FP-a sequence. The core C/EBP family- and the core PU.1 binding sites of the C/EBP- and PU.1 oligonucleotides are aligned with the corresponding sequences of FP-am44 and FP-am41, respectively. The point mutations in FP-am44 and FP-am41 are in lower-case typeface and are at the head of arrows leading from the corresponding sites on the wild-type FP-a sequence. **B** and **C**, EMSAs using 0.5 μg of nuclear extract from RAW 264.7 cells incubated overnight in medium containing fresh 10% (v/v) FCS; **B:** Nuclear extract was incubated with 1 ng of FP-a or FP-am44 probe in the absence (-) or presence (+) of 25 ng (FP-a, dFP-c, FP-am44, PU.1) or 100 ng (αp3a) of competitor (Comp.). Excess probe migrated as shown (free probe), and retarded nucleoprotein complexes a1-a4 are marked. The variable a5 complex formed with FP-a probe, and non-specific complexes formed with FP-am44 probe are not marked. **C:** Nuclear extract was incubated with 1 ng of either FP-a or FP-am41 probe, in the absence (-) or presence (+) of 25 ng of PU.1, FP-am41 or FP-a competitor (Comp.), as shown. Uncomplexed excess probe (free probe) and retarded complexes a1-a4 are as shown.

Coding-strand sequences of oligonucleotides:

FP-a: CCCAGATTCCATTTCCTCATCTGCTAGGG

FP-am41: CCCAGATTCCATTTCCTCTTTTGCTAGG

FP-am44: CCCAGATTCCAATTACACAACTGCTAGG

dFP-c: ACCCTGGGTTGTGTAATATCCA

PU.1: ATAACCTCTGAAAGAGGAACTTGGTTAGGT (Klemsz *et al*, 1990)

αP3a: GATCCAAACCAGCCAATGAGAACTGCTCCA (MacLeod *et al*, 1991)

are unable to form with the FP-am41 probe, while formation of complex a4 is unaffected (figure **11.6c**).

The proposed C/EBP family binding site of oligo dFP-c was then superimposed over that of FP-a, creating a second variant, FP-am44 (figure **11.6a**). Using FP-am44 probe with RAW 264.7 cell nuclear extracts, complex a4 (which is unrelated to C/EBP (figures **11.2b, 11.2c, 11.3**)) was unable to form, but complexes a1 to a3 were retained (figure **11.6b**). In the reciprocal experiment, FP-am44 competitor competed for complexes a1-a3, but not for complex a4 formed with FP-a probe and RAW 264.7 cell nuclear extracts. Further, the a3 complexes formed with probes FP-a and FP-am44 co-migrate (figure **11.6b**), suggesting that the same C/EBP-related nucleoprotein complexes are in fact formed with oligos FP-a, dFP-c, pFP-c, FP-d, C/EBP and mGPE-1.

Although I have not used the UV crosslinking technique to study the molecular size of protein components of complex 3/a3 formed with FP-a probe in EMSAs, the possible identity of the C/EBP-related proteins present in this complex is addressed in the Discussion.

11.4 PRELIMINARY IDENTIFICATION OF PU.1 AND Spi-B BINDING TO THE FP-a PROBE, AND DELINIATION OF THE DNA SITE TO WHICH THEY BIND.

11.4.1 FP-a contains a PU.1/ Spi-B binding site.

In addition to the C/EBP family binding site discussed above, FP-a also contains a purine-rich sequence which is similar to that bound by two members of the ETS family of proto-oncogenic transcription factors, namely the macrophage and B cell-specific transcription factor PU.1 (figure **11.6a**) (Goebel *et al*, 1990; Klemsz *et al*, 1990), and Spi-B, which is hemopoietic cell-specifically expressed (Ray *et al*, 1992). A competitor containing a known PU-1- and Spi-B binding site (Klemsz *et al*, 1990; Ray *et al*, 1992) specifically competes for complex a4 , but not complexes a1-a3, formed with RAW 264.7 cell nuclear extracts and FP-a probe (figure **11.2a**).

Complex a4 is also formed with FP-a probe and MEL cell nuclear extracts (figure **11.7a**), as would be expected, as PU-1 is expressed in MEL cells as a consequence of viral enhancer insertion at the Spi-1 (PU-1) locus, and Spi-B is expressed in MEL cells (Moreau-Gachelin,F. *et al*, 1989; Ray *et al*, 1992). Also as would be expected, complex a4 is not formed with STO cell nuclear extracts (figure **11.7b**), as PU-1 and Spi-B are hemopoietic cell-specifically expressed (a faint complex formed with FP-a probe and STO nuclear extracts does not compete with PU-1 competitor (figure **11.7b**)). Further, RAW 264.7- and MEL cell nuclear extracts both form a complex with PU-1 probe that is cross-competed by 25-fold excess of FP-a competitor (figure **11.7c**) and which co-migrates with complex a4 formed with RAW 264.7- or MEL cell nuclear extracts and FP-a probe (figure **11.7d**).

The FP-a purine-rich sequence is not completely homologous to the consensus PU-1/ Spi-B recognition sequence (figure **11.6a**). However, the mutations in the variant FP-a oligonucleotide probe FP-am41, which abolish C/EBP family binding (figure **11.6c**), introduce a characteristic PU-1/ Spi-B binding motif into FP-am41 (figure **11.6a**). EMSA studies with variant FP-am41 probe demonstrate that complex a4 is still obtained and that FP-am41, PU.1 and FP-a oligos cross-compete. Conversely, variant oligo FP-am44 contains two base changes within the FP-a core GAGGAA PU-1/Spi-B binding motif (figure **11.6a**), and complex a4 is no longer formed (figure **11.6b**).

11.4.2 UV crosslinking studies of complex a4 formed with FP-a probe.

Because of the overlapping nature of the C/EBP family and ETS family binding sites in the FP-a sequence (figures **11.5a** and **11.6a**), three of the BrdU substitutions on the coding strand of the BrdU-substituted FP-a probe used to study C/EBP family binding to FP-a are also within the PU.1/SpiB core binding site (figure **11.5a**). The RAW 264.7 cell nuclear protein components of complex a4 formed with the BrdU-substituted FP-a probe were therefore also resolved by SDS-PAGE after UV-crosslinking (figure **11.5b**). Complex a4 contains two distinct protein components, which are of different molecular size to the C/EBP family proteins present in complexes a1 and a2 (figure **11.5b**). After subtracting the molecular size of the probe

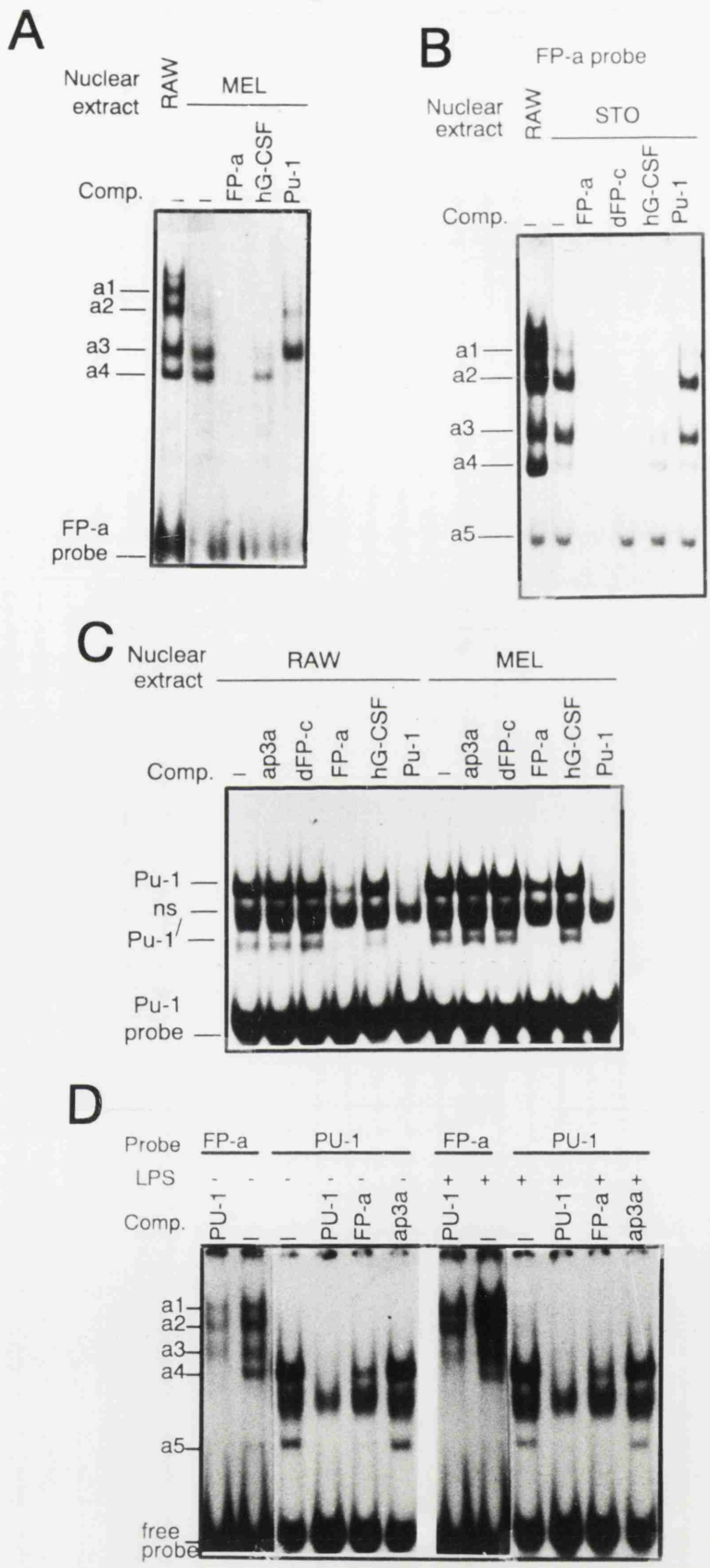

A
Nuclear extract
RAW
MEL
Comp.
–
–
FP-a
hG-CSF
Pu-1
a1
a2
a3
a4
FP-a probe
B
FP-a probe
Nuclear extract
RAW
STO
Comp.
–
–
FP-a
dFP-c
hG-CSF
Pu-1
a1
a2
a3
a4
a5
C
Nuclear extract
RAW
MEL
Comp.
–
ap3a
dFP-c
FP-a
hG-CSF
Pu-1
–
ap3a
dFP-c
FP-a
hG-CSF
Pu-1
Pu-1
ns
Pu-1/
Pu-1 probe
D
Probe
FP-a
PU-1
FP-a
PU-1
LPS
- - - - - - + + + + + +
Comp.
PU-1
–
–
PU-1
FP-a
ap3a
PU-1
–
–
PU-1
FP-a
ap3a
a1
a2
a3
a4
a5
free probe

Figure 11.7: EMSA studies of ETS family nucleoprotein binding to MIP1α promoter site FP-a.

EMSA anslysis of RAW 264.7-, MEL- and STO cell nuclear protein binding to MIP1α promoter site FP-a probe and to PU.1 probe. Specific retarded nuclear protein complexes a1 to a5 (FP-a probe) and PU.1 and PU.1' (PU.1 probe), a non-specific complex (ns) formed with the PU.1 probe, and excess uncomplexed FP-a and PU.1 probes (free probe) are as marked. **A:** 0.5 μg of nuclear extracts, from either RAW 264.7 cells incubated overnight in SLM containing fresh 10% (v/v) FCS (RAW) or from MEL cells (MEL), were incubated in EMSAs with 1 ng of FP-a probe in the absence (-) or presence (+) of 25 ng of FP-a, hG-CSF or PU.1 competitor (Comp.). **B:** EMSAs in which 0.5 μg of nuclear extract, from either RAW 264.7 cells incubated overnight in medium containing fresh 10% (v/v) FCS or from STO cells, was incubated with 1 ng of probe FP-a in the absence (-) or presence (+) of 25 ng of FP-a, dFP-c, hG-CSF or PU.1 competitor (Comp.). A faint complex formed with FP-a probe and STO cell nuclear extracts, which migrates similarly to complex a4 but which does not compete with PU.1 competitor, is visible but is not labelled. Uncomplexed excess probe has migrated off the end of the gel. **C:** EMSAs in which 1 μg of nucleoprotein, from RAW 264.7 cells incubated for 24 hours in medium containing fresh 10% (v/v) FCS or from MEL cells, was incubated with 1 ng of PU.1 probe, in the absence (-) or presence (+) of 25 ng (FP-a, PU.1) or 50 ng (αp3a, dFP-c, hG-CSF) competitor. **D:** EMSAs in which 1 μg of nuclear extracts from RAW 264.7 cells incubated for 22 hours in medium containing fresh 10% (v/v) FCS in the absence (-) or presence (+) of 2 μg/ml LPS, was incubated with 1 ng of either FP-a or PU.1 probe in the absence (-) or presence (+) of 25 ng (PU.1, FP-a) or 100 ng (αp3a) of competitor oligonucleotide.

Coding-strand sequences of oligonucleotides:
FP-a: CCCAGATTCCATTTCCTCATCTGCTAGGG
dFP-c: ACCCTGGGTTGTGTAATATCCA
PU.1: ATAACCTCTGAAAGAGGAACTTGGTTAGGT (Klemsz *et al*, 1990)
αP3a: GATCCAAACCAGCCAATGAGAACTGCTCCA (MacLeod *et al*, 1991)
hG-CSF: GGATCAGAGATTCCACAATTTC

(18 kDa, figure **11.5b**), the two distinct protein components of complex a4 have predicted molecular sizes of ~37 kDa and ~30 kDa respectively, consistent with the molecular sizes of PU.1 (~36 kDa) and SpiB (~33 kDa) deduced from the published amino acid sequences (Klemsz *et al*, 1990; Ray *et al*, 1992). Together with the data presented above, this data suggests that site FP-a of the MIP1α promoter can bind C/EBP family- and PU.1/SpiB at overlapping sites.

11.5 CELL-SPECIFIC NUCLEOPROTEIN BINDING TO THE MIP1α PROXIMAL PROMOTER FP-a, pFP-c, dFP-c and FP-d SITES.

11.5.1 Overview.

The pM-160 proximal MIP1α promoter is ~25-fold more active in transient transfection assays in RAW 264.7 cells compared to STO- or MEL cells. However, nuclear extracts from these three cell types revealed a similar pattern of transcription factor binding to this region in *in-vitro* footprinting experiments (figure **11.1**). Hence, nuclear extracts from these three cell-types were used in EMSAs in order to investigate differences in nucleoprotein binding to the proximal promoter in more detail. Results for site FP-b are presented in section **11.7**.

11.5.1 FP-a site.

FP-a probe binding by MEL cell nuclear extracts revealed the presence of PU-1/ SpiB-like complex a4 and C/EBP family-containing complexes a1 to a3 (figure **11.7a**: complex a1 is very faint, and is best seen in the lane competed by the PU.1 competitor). STO nuclear extracts yielded complexes a1, a2 and a3, but although an STO nucleoprotein complex which co-migrates with a4 is detected, it is not competed out by PU-1 competitor (figure **11.7b**), and is therefore distinct from the a4 complex detected with RAW 264.7 and MEL cell nuclear extracts (figures **11.2b** and **11.7a**). Nuclear extracts from unstimulated RAW 264.7, STO- and MEL cells each form significantly less of C/EBP-related complex a1 than of C/EBP-related complexes a2 and a3 (figures **11.2b**, **11.4a**, **11.7a** and **11.7b**).

11.5.2 pFP-c site.

In each case, the incubation of RAW 264.7-, MEL- or STO cell nuclear extracts with pFP-c probe revealed the presence of C/EBP-related complexes 1, 2 and 3 (figure **11.8a**), though the ratio in the abundance of complexes 1, 2 and 3 was different in different cell types, in that with RAW 264.7- and STO cell nuclear extracts relatively high levels of complex 1 were formed, whereas with MEL nuclear extracts, relatively high levels of complex 3 were formed (figure **11.8a**). Further, with RAW 264.7 cell nuclear extracts, three faster-migrating specific complexes were obtained with this probe (figures **11.4b** and **11.8a**), each of which is competed out by pFP-c or FP-a competitors (figure **11.4b**). However, of these three further complexes, MEL cell nuclear extracts contain only the two fastest migrating, while STO cell nuclear extracts contain none of them (figure **11.8a**).

11.5.3 dFP-c site.

Nuclear extracts from unstimulated RAW 264.7 cells, MEL cells and STO cells each contain proteins able to form C/EBP-related complexes 1, 2 and 3 with dFP-c probe, though formation of complex 1 is in each case very faint compared to complexes 2 and 3 (figure **11.8a**). No other specific nucleoprotein binding to this probe is detectable using these extracts.

11.5.4 FP-d site.

RAW 264.7 cell nuclear extracts contain C/EBP family proteins able to form complexes 1, 2 and 3 with FP-d probe, though in EMSAs the binding to this site is weaker than to the other MIP1α promoter C/EBP family binding sites characterized in this study (figure **11.4** and discussed earlier).

When MEL cell nuclear extracts are incubated with FP-d probe, four specific complexes are obtained (bands 1 to 4, MEL cell nuclear extracts, figure **11.8b**). Though the resolution of these complexes is not very clear in figure **11.8b**, C/EBP-like binding is detectable, as complex 3 is specifically competed by FP-a and C/EBP competitors, but not by κBpd competitor (figure **11.8b**, MEL cell nuclear extracts, band 3). Abundant binding of three other complexes (figure **11.8b**, MEL cell nuclear extracts, bands 1, 2 and 4) is also obtained, and the proteins forming these complexes are not related to C/EBP, as they are not competed by FP-a or C/EBP competitors.

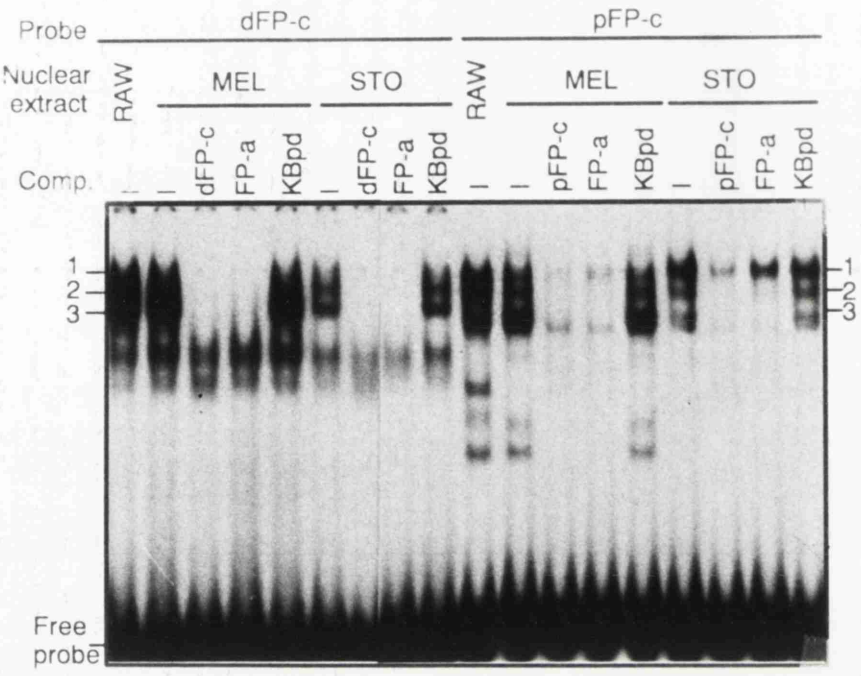
Figure 11.8: EMSA detection of cell-specific binding of C/EBP family- containing nucleoprotein complexes to proximal MIP1α promoter sites.

EMSA anslysis of RAW 264.7, STO and MEL cell nuclear protein binding to MIP1α promoter sites dFP-c, pFP-c and FP-d. **A:** 0.5μg of nuclear extract from unstimulated RAW 264.7 (RAW)-, MEL- or STO cells was incubated in EMSAs with 1 ng of dFP-c or pFP-c probe in the absence (-) or presence (+) of 25 ng (dFP-c, pFP-c, FP-a) or 100 ng (κBpd) of competitor (Comp.). Specific C/EBP-related retarded complexes 1-3 are labelled, though complex 1 formed with dFP-c probe is very faint. Three faster-migrating C/EBP-related complexes formed with pFP-c probe and unstimulated RAW 264.7 cell nuclear extracts, and two such complexes formed with this probe and MEL cell nuclear extracts (see text) are visible but not labelled. Two non-specific retarded complexes formed with dFP-c probe and each of the nuclear extracts are visible but not labelled. Excess uncomplexed dFP-c and pFP-c probe (free probe) is labelled. **B:** EMSAs in which 0.5 μg of nuclear extract from MEL- or STO cells was incubated with 1 ng of FP-d probe in the absence (-) or presence (+) of 25 ng (FP-d, FP(d+e), FP-a) or 100 ng (κBpd) of competitor (Comp.). Specific retarded complexes 1-4 formed with MEL cell nuclear extracts and 1-3 formed with STO cell nuclear extracts are labelled, as is excess uncomplexed FP-d probe (free probe).

Coding-strand sequences of oligonucleotides:

FP-a: CCCAGATTCCATTTCCTCATCTGCTAGGG
pFP-c: TCCATCATGACACCATTGCTGTG
dFP-c: ACCCTGGGTTGTGTAATATCCA
FP-d: GGGCCCCTGTGGTCACTGTGGACCCT
C/EBP: GCTGCAGATTGCGCAATCTGCAGC (Landschultz *et al*, 1988)
κBpd: CAACGGCAGGGGAATTCCCCTCTCCTT (Ballard *et al*, 1990)
FP(d+e): AGACTTATATCTCAGAGATGCTATTCTTAGATATCCTGG
GCCCCTGTGGTCACTGTGGACCCTGG

A


Probe
dFP-c
pFP-c
Nuclear extract
RAW
MEL
STO
RAW
MEL
STO
Comp.
–
–
dFP-c
FP-a
KBpd
–
dFP-c
FP-a
KBpd
–
–
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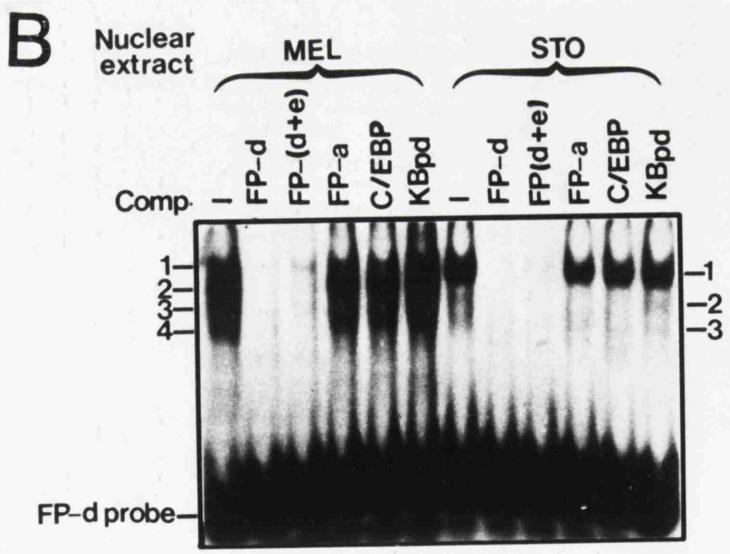
–
pFP-c
FP-a
KBpd
1
2
3
1
2
3
Free probe

B


Nuclear extract
MEL
STO
Comp
–
FP-d
FP-(d+e)
FP-a
C/EBP
KBpd
–
FP-d
FP(d+e)
FP-a
C/EBP
KBpd
1
2
3
4
1
2
3
FP-d probe

Incubation of STO cell nuclear proteins with FP-d probe also reveals C/EBP-related binding (figure **11.8b**, STO cell nuclear extracts, bands 2 and 3), but also formation of an uncharacterised more abundant slower-migrating complex which is unrelated to C/EBP, as it is not competed by FP-a or C/EBP competitors (figure **11.8b**, STO cell nuclear extracts, band 1).

Hence, RAW 264.7 cell nuclear extracts form three C/EBP-related nucleoprotein complexes with FP-d probe, while with the same probe, only one C/EBP-related complex is detectable using MEL cell nuclear extracts, and only two C/EBP-related complexes detected using STO nuclear extracts (figures **11.4** and **11.8b**). The explanation for this discrepancy is probably simply that FP-d probe binds C/EBP-related proteins fairly weakly, and C/EBP-related complexes formed with FP-d and MEL or STO nuclear extracts are obscured by the more abundant non-C/EBP-related complexes formed with this probe and nuclear extracts from these cell-types.

These studies show that the clearest cell-specific differences exist in the formation of nucleoprotein complexes with probe FP-d, and as the pM-160 promoter is inactive in STO- and MEL-cells but strongly active in RAW 264.7 cells, this suggests that the FP-d site may be involved in the negative regulation of MIP1α promoter constructs in functional transient transfection assays in STO- and MEL cells. Although PU.1/ Spi-B is absent in STO cell nuclei, its presence is clearly not sufficient to activate the MIP1α promoter, as the promoter is not active in PU.1/ Spi-B -containing MEL cells. Although similar patterns of C/EBP-related binding are obtained with the above probes and unstimulated RAW 264.7-, MEL- and STO cell nuclear extracts, some differences do exist. The contribution of such differences to the cell-specificity of MIP1α promoter expression is not immediately obvious, and will be addressed in the Discussion in light of further results presented below.

11.6 INDUCIBLE NUCLEOPROTEIN BINDING TO MIP1α PROMOTER C/EBP FAMILY BINDING SITES.

11.6.1. FP-a.

In contrast to nuclear extracts from RAW 264.7 cells incubated overnight in medium containing fresh 10% (v/v) FCS (figures **11.6b, 11.6c, 11.9a, 11.9b,** lanes 1 and figure **11.3**), nuclear extracts from MEL cells, STO cells and unstimulated RAW 264.7 cells contain similarly low levels of protein(s) able to form complex a1 with FP-a probe (**11.2b, 11.4a** and **11.9**).

When binding to FP-a probe is analysed using nuclear extracts prepared from RAW 264.7 cells incubated with LPS over a timecourse, binding of complex a1 (and to a lesser extent a2) transiently increases and then decreases over the timecourse (figure **11.9c**). Comparison of a1 binding (figure **11.9c**) and endogeous MIP1α mRNA levels (figure **9.2a**) reveals that a1 is low in control cells which have basal levels of MIP1α mRNA; a1 binding is high six hours after LPS stimulation, when MIP1α mRNA levels are high; and a1 binding is low again sixteen hours after LPS stimulation when MIP1α mRNA levels are decreasing. In contrast, C/EBP-family binding in complex a3 gradually diminishes over the sixteen hour timecourse (figure **11.9c**), implicating C/EBP-related complex a1 (and a2) in the LPS response of the MIP1α promoter, and suggesting that C/EBP-related complex a3 may be a negative transcriptional regulator. Levels of the proposed PU.1/Spi-B complex a4 formed with the FP-a probe do not change over this timecourse (figure **11.9c**), suggesting that, ignoring possible post-translational modifications that affect transactivation but not DNA binding, the PU.1/ Spi-B complex is not involved in the LPS response. This is further supported by studies with the PU.1 probe (figure **11.7d**) which show no change in PU-1/Spi-B binding upon LPS stimulation of RAW 264.7 cells.

Although detailed studies of nucleoprotein binding to the FP-a- or other MIP1α promoter C/EBP family binding sites have not been undertaken in serum stimulated RAW 264.7 cells, there is evidence that the C/EBP family-containing a1 complex binding to the FP-a probe is serum-responsive. For example, a1 binding is very weak in nuclear extracts from unstimulated RAW 264.7 cells (figures **11.2b, 11.4a, 11.9c**), whereas it is significantly increased when cells are incubated overnight in medium containing fresh 10% FCS (figures **11.6b, 11.6c, 11.9a, 11.9b,** lanes 1 and figure **11.3**).

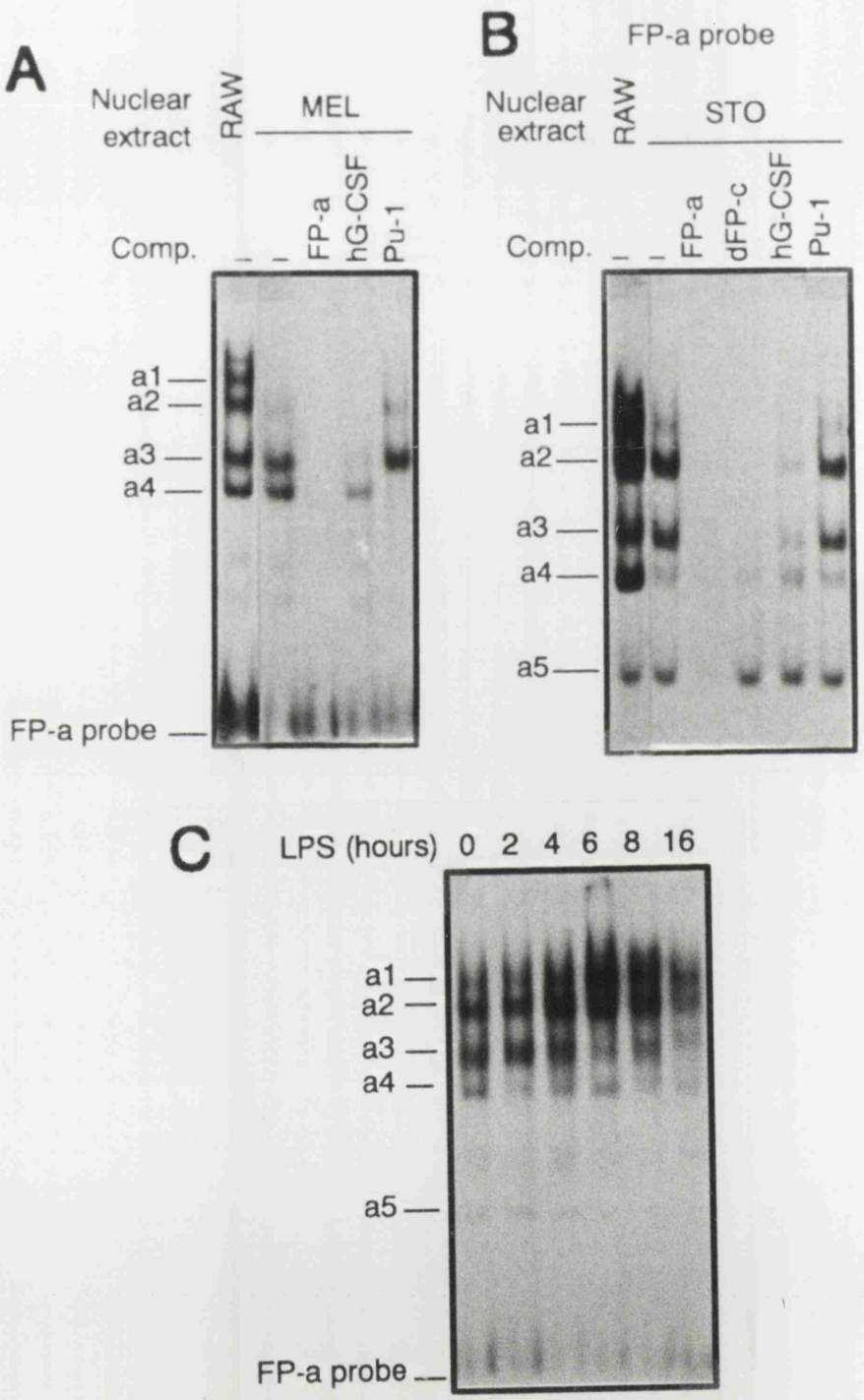

A
Nuclear extract
RAW
MEL
Comp.
FP-a
hG-CSF
Pu-1
a1
a2
a3
a4
FP-a probe
B
FP-a probe
Nuclear extract
RAW
STO
Comp.
FP-a
dFP-c
hG-CSF
Pu-1
a1
a2
a3
a4
a5
C
LPS (hours) 0 2 4 6 8 16
a1
a2
a3
a4
a5
FP-a probe

Figure 11.9: EMSA studies of cell-specific and inducible nucleoprotein binding to the proximal MIP1α promoter site FP-a.

EMSA anslysis of RAW 264.7-, MEL- and STO cell nucleoprotein binding to MIP1α promoter site FP-a. C/EBP-related complexes a1-a3, PU.1/Spi-B-related complex a4, variably-formed complex a5 (when visible) and uncomplexed excess FP-a probe are all labelled. **A:** 0.5 μg of nuclear extracts, from either RAW 264.7 cells incubated overnight in SLM containing fresh 10% (v/v) FCS (RAW) or from MEL cells (MEL), were incubated in EMSAs with 1 ng of FP-a probe in the absence (-)or presence (+) of 25 ng of FP-a, hG-CSF or PU.1 competitor (Comp.). Complex a1 formed with MEL cell nuclear extracts is very faint, but is present and is most visible in the lane competed for by PU.1 competitor. **B:** EMSAs in which 0.5 μg of nuclear extract, from either RAW 264.7 cells incubated overnight in medium containing fresh 10% (v/v) FCS or from STO cells, was incubated with 1 ng of FP-a probe in the absence (-) or presence (+) of 25 ng of FP-a, dFP-c, hG-CSF or PU.1 competitor (Comp.). A faint complex formed with FP-a probe and STO cell nuclear extracts,which migrates similarly to complex a4 but which does not compete with PU.1 competitor, is visible but is not labelled. Uncomplexed excess probe has migrated off the end of the gel. **C:** EMSAs in which 0.5 μg of nucleoprotein, from either unstimulated RAW 264.7 cells (0, lane 1) or RAW 264.7 cells incubated for the indicated times (in hours) in RAW CM containing 2 μg/ml LPS and fresh 1% (v/v) FCS, was incubated with 1 ng of FP-a probe.
Coding-strand sequences of oligonucleotides:
FP-a: CCCAGATTCCATTTCCTCATCTGCTAGGG
dFP-c: ACCCTGGGTTGTGTAATATCCA
PU.1: ATAACCTCTGAAAGAGGAACTTGGTTAGGT (Klemsz *et al*, 1990)
hG-CSF: GGATCAGAGATTCCACAATTTC (Shannon *et al*, 1990)

In addition to macrophages, Balb/C 3T3 fibroblasts are functionally LPS-responsive, and erythroid cells display cell-surface LPS receptors (Tannenbaum *et al*, 1990; Chen *et al*, 1992). However, in EMSAs with FP-a probe and nuclear extracts from LPS stimulated MEL or STO cells, the nucleoprotein binding pattern is identical to that formed with the same probe and nuclear extracts from unstimulated STO- or MEL cells (therefore data not shown). Further, in EMSAs with FP-a probe and nuclear extracts from NIH 3T3 fibroblasts that have been serum starved and then serum stimulated, no observable complex formation with probe FP-a is obtained (therefore data not shown).

11.6.2 pFP-c, dFP-c, FP-d.

No timecourse of RAW 264.7 cell C/EBP family binding to these MIP1α promoter C/EBP family binding sites was carried out as, given the data presented above, it is reasonable to assume that C/EBP-like binding to these sites will change over the timecourse in response to LPS in a similar manner to that described for FP-a probe. This is further supported by the data shown in figure **11.4a**, in which nucleoprotein binding to these sites is compared in nuclear extracts from RAW 264.7 cells that are either unstimulated or LPS stimulated for 6 hours. In each case, after 6 hours' stimulation, complexes 1 and 2 are enhanced and complex 3 diminished in comparison to the unstimulated cells. Further, nuclear extracts from RAW 264.7 cells stimulated for 4 hours with LPS were used in the binding studies shown in figure **11.4b**, and the shift in binding intensity from complex 3 to complexes 1 and 2 is intermediate to that obtained with nuclear extracts from unstimulated- and 6 hour LPS stimulated RAW 264.7 cells. Hence, all the data supports the idea that C/EBP-related binding of complex a1 and complexes 1 to MIP1α promoter sites FP-a, dFP-c, pFP-c and FP-d peaks at approximately 6 hours after LPS stimulation, which is when maximal LPS-stimulated endogenous MIP1α mRNA levels are obtained.

A further point of note is that, while for sites FP-a, dFP-c, C/EBP and mGPE-1, complex a1/1 is in very low abundance compared to complexes a2/2 and a3/3 in unstimulated RAW 264.7 cells, for sites pFP-c and FP-d, complex 1 is in relatively high abundance (compared to complexes 2 and 3) in nuclear extracts from such

unstimulated cells (figure **11.4a**). This point will be addressed in the Discussion section.

11.7 MIP1α PROXIMAL PROMOTER SITE FP-b BINDS NF-κB AND RELATED TRANSCRIPTION FACTORS.

MIP1α promoter site FP-b (figure **11.1c**) contains a GGGAA motif which is homologous to the most highly-conserved half site of a consensus NF-κB recognition sequence (Baeuerle, 1991) (figure **11.10a**). As LPS stimulation of macrophages is known to enhance the levels of nuclear DNA-binding NF-κB (Vincenti *et al*, 1992), nuclear extracts from LPS stimulated RAW 264.7 cells were therefore incubated with FP-b probe or with κBpd probe, which contains a palindromic NF-κB binding site (Ballard *et al*, 1990). Two prominent retarded complexes were obtained with both of these probes, K1 and K2 with κBpd probe, and b1 and b2 with FP-b probe (figure **11.10b**).

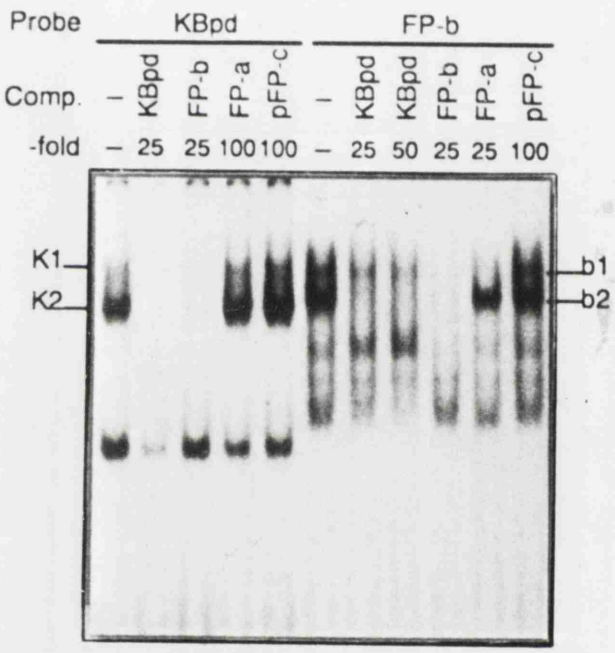
Complexes K2 and b2 are both completely abolished by a 25-fold excess of FP-b and κBpd competitors, suggesting these complexes are equivalent (figure **11.10b**). Complex K1 is abolished by a 25-fold excess of FP-b and κBpd competitors, while complex b1 is abolished by a 25-fold excess of FP-b competitor but only weakly competed by a 50-fold excess of κBpd competitor (figure **11.10b**). This information alone would suggest that K1 and b1 are equivalent complexes, but with higher binding affinity for FP-b- than κBpd probe. However, competitor FP-a competes strongly for complex b1 but does not compete for complex K1 (figure **11.10b**), which suggests that b1 and K1 are distinct but related nucleoprotein complexes.

The fact that competitor pFP-c, which is the highest affinity MIP1α promoter C/EBP family binding site (see figure **11.4b**), does not compete for any of the complexes formed with κBpd or FP-b probes (figure **11.10b**), shows that, though FP-a competitor competes for complex b1, complex b1 is not related to C/EBP. In the reciprocal experiment, FP-b competitor does not observably compete for any complex formed with FP-a probe (figure **11.2b**), but this may be attributed to the high levels of *in-vitro* binding of C/EBP family proteins to the FP-a probe which obscure

A

FP-b (c)	CTTAAAATTTTCCCTCCTCAC
κBμd	gggaaTTCCC
FP-a	tccaTTTCCt

B



C

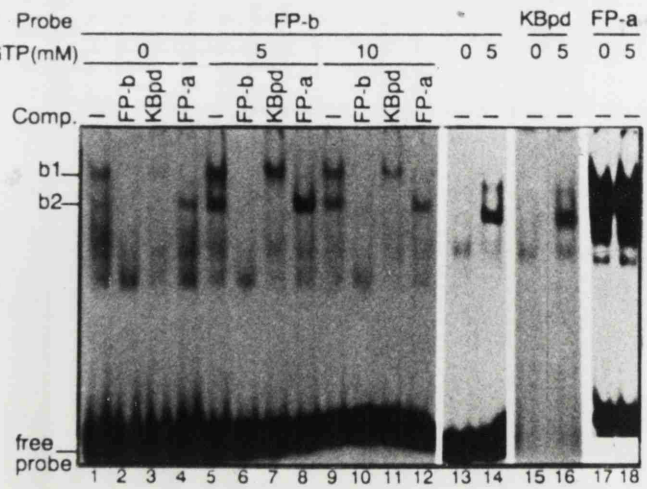


Figure 11.10: EMSA detection of NF-κB-related nucleoprotein complexes binding to the proximal MIP1α promoter site FP-b.

EMSA anslysis of RAW 264.7 cell nuclear protein binding to MIP1α promoter site FP-b. **A:** Sequence comparison of MIP1α promoter site FP-b, with the most homologous regions of κBpd, which is an oligonucleotide containing a palindromic NF-κB binding site, and of MIP1α promoter site FP-a. (c), coding sequence. Upper case letter denote identity and lower case letters non-identity, respectively, of FP-a and κBpd sequences with FP-b. **B:** EMSAs in which 2 μg of nuclear extract from 8 hr LPS stimulated RAW 264.7 cells was incubated with 1 ng of either κBpd- or FP-b probe in the absence (-) or presence (+) of 25 ng or 100 ng of competitor (Comp.), as shown. Specific retarded nucleoprotein complexes K1 and K2 formed with κBpd probe, and b1 and b2 formed with FP-b probe are labelled. Faster migrating complexes formed with κBpd- or FP-b probe are either non-specific, are variably formed and/or their significance is unclear, and these complexes are not labelled. Excess uncomplexed FP-b and κBpd probes have migrated off the bottom of the gel. **C:** EMSAs in which 0.5 μg of nuclear extract from 6 hr LPS-stimulated (2 μg/ml) RAW 264.7 cells was incubated with either FP-b, κBpd or FP-a probe in the absence (-) or presence (+) of 25 ng of the indicated competitor (Comp.). EMSAs were performed in the presence or absence of GTP:- lanes 1-4, 13, 15 and 17: no added GTP; lanes 5-8, 14, 16, 18: GTP added to a final concentration of 5 mM; lanes 9-12, GTP added to a final concentration of 10 mM. Specific retarded nucleoprotein complexes b1 and b2 formed with FP-b probe in lanes 1-12 are labelled. GTP-sensitive b1 and b2 complexes formed with FP-b probe and K1 and K2 formed with κBpd probe, and GTP-insensitive complexes a1-a4 formed with FP-a probe are visible (lanes 13-18) but are not labelled. Variably-formed and non-specific complexes formed with FP-b and κBpd probes are visible but not labelled. Uncomplexed excess FP-b, κBpd and FP-a probe is labelled (free probe), except in lanes 15 and 16, where free κBpd probe has run off the end of the gel.

Coding-strand sequences of oligonucleotides:
FP-a: CCCAGATTCCATTTCCTCATCTGCTAGGG
pFP-c: TCCATCATGACACCATTGCTGTG
κBpd: CAACGGCAGGGGAATTCCCCTCTCCTT (Ballard *et al*, 1990)
FP-b: CTTAAAATTTTCCCTCCTCAC

other similarly-migrating complexes binding to this site. A further possible explanation is that FP-a probe binds a *subset* of the proteins that form complex b1 (several uncharacterised faster-migrating complexes form with FP-a probe, eg. complex a5, see above), correlating with the possibility that complex b1 and complex K1 do not contain exactly the same protein(s), but share some of the same components, none of which bind FP-a probe.

These experiments suggested that complexes b2 and K2 are in fact due to NF-κB binding. As *in-vitro* NF-κB binding is enhanced in the presence of mM concentrations of GTP, binding to FP-b and κBpd probes was further investigated with respect to GTP concentration. As shown in figure **11.10c**, formation of both complexes b1 and b2 with FP-b probe and K1 and K2 with κBpd probe is enhanced in the presence of 5mM GTP. In contrast, and as a control, observed *in-vitro* binding of C/EBP- and Ets-related complexes to FP-a probe is unaffected by the presence of the same concentration of GTP (figure **11.10c**). Further, a 25-fold molar excess of κBpd competitor competes out complex b1 formed with FP-b probe in the absence of GTP, but does not compete in the presence of GTP (figure **11.10c**), suggesting that the difference in affinity between FP-b- and κBpd probes for the protein(s) forming complex b1 is greater in the presence- compared to the absence of GTP. Again, this information suggests that complex b2 is NF-κB, while complex b1 may be related to NF-κB. All further EMSAs using the FP-b probe and RAW 264.7 cell nuclear extracts were conducted in the presence of 5 mM GTP, unless otherwise stated.

LPS stimulation of RAW 264.7 cells greatly enhances the presence of nuclear DNA binding NF-kB (Vincenti *et al*, 1992). Hence, if complex b2 formed with FP-b probe is NF-κB, there should be increased formation of complex b2 with FP-b probe and nuclear extracts from LPS-stimulated- compared to unstimulated RAW 264.7 cells. As shown in figure **11.11a**, this is indeed the case. Formation of complex K2 with κBpd probe is similarly enhanced with nuclear extracts from LPS-stimulated-compared to unstimulated RAW 264.7 cells (figure **11.11b**). No timecourse of LPS induction of NF-κB binding in RAW 264.7 cells was conducted, though LPS

A


Nuclear Extract
RAW
STO
LPS
Comp.
FP-b
KBpd
FP-a
ap3a
b1
b2

B

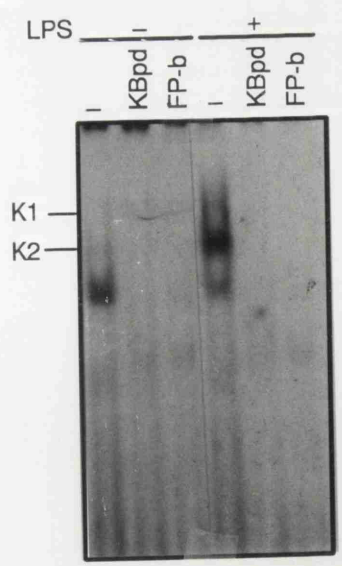

LPS
KBpd
FP-b
K1
K2

Figure 11.11: LPS-responsiveness and cell-specificity of NF-κB-related nucleoprotein complexes binding to FP-b and κBpd probes in EMSAs.

EMSA anslysis, in the presence of 5 mM GTP, of nucleoprotein binding to MIP1α promoter site FP-b and palindromic NF-κB binding site κBpd, using nuclear extracts from STO cells and unstimulated and LPS-stimulated RAW 264.7 cells. **A:** EMSAs in which 1 μg of nuclear extract from STO cells or from unstimulated (-) or 6 hr LPS stimulated (+) RAW 264.7 cells (RAW) was incubated with FP-b probe in the absence (-) or presence (+) of 25 ng (FP-b, κBpd, FP-a) or 100 ng (αp3a) of competitor (Comp.). Specific retarded nucleoprotein complexes b1 and b2 are labelled. Non-specific and variably-formed faster migrating complexes are visible but not labelled, and uncomplexed excess FP-b probe has migrated off the end of the gel. **B:** EMSAs in which 1 ng of κBpd probe was incubated with 1 μg of nuclear extract from unstimulated (-) or 6 hr LPS-stimulated (+) RAW 264.7 cells in the absence (-) or presence (+) of 25 ng of κBpd or FP-b competitor. Specific retarded nucleoprotein complexes K1 and K2 are labelled, faster-migrating non-specific or variably-formed nucleoprotein complexes are visible but not labelled, and uncomplexed excess κBpd probe has migrated off the end of the gel.
Coding-strand sequences of oligonucleotides:
FP-a: CCCAGATTCCATTTCCTCATCTGCTAGGG
κBpd: CAACGGCAGGGGAATTCCCCTCTCCTT Ballard *et al*, 1990)
FP-b: CTTAAAATTTTCCCTCCTCAC
αP3a: GATCCAAACCAGCCAATGAGAACTGCTCCA (MacLeod *et al*, 1991)

activation of macrophages reportedly leads to prolonged nuclear localization of NF-κB (Cordle *et al*, 1993).

The fact that complex b1 formed with probe FP-b is constitutively formed, while complex b2 formed with the same probe is LPS-inducible suggests that these complexes may compete for binding at site FP-b, implying that complex b2 might displace complex b1 bound at FP-b, as part of the LPS-responsiveness of the MIP1α promoter. To investigate this possibility, preliminary EMSA experiments were performed using limiting amounts of FP-b probe and LPS-induced RAW 264.7 cell nuclear extracts. In EMSAs under standard conditions, complex b2 formation is favoured over complex b1 formation (figure **11.12**). However, addition of additional fresh 1mM DTT to the binding buffer results in the favouring of complex b1 formation over that of complex b2 (figure **11.12**). This suggests that optimal binding conditions for complexes b1 and b2 to FP-b differ with respect to REDOX conditions, supporting the idea that binding of these complexes to FP-b is mutually exclusive *in-vivo*, and consistent with inolvement of complex b1 in basal- and complex b2 in induced MIP1α promoter activity.

This data indicates that FP-b binds NF-κB (complex b2), and as formation of complex b1 is GTP-dependent and is partially competed by κBpd competitor, it suggests that complex b1 may contain NF-κB-related protein(s). Furthermore, it suggests that in macrophages complex b1 may contribute to the basal level of MIP1α promoter activity, whereas complex b2 is involved in the LPS-response. As neither complex b2 (NF-κB) nor complex b1 is formed with FP-b probe and nuclear extracts from continuously cycling STO fibroblasts (figure **11.11a**) or MEL cells (data not shown, as not even non-specific complexes were formed), this suggests that presence (RAW 264.7 cells) or absence (STO- and MEL cells) may contribute to the cell-specificity of MIP1α promoter activity.

CHAPTER 12: THE MIP1α PROMOTER DISTAL (-160 bp to -220 bp) REGULATORY ELEMENT.

Figure 11.12: REDOX-responsive binding of NF-κB-related nucleoprotein complexes to FP-b probe in EMSAs.

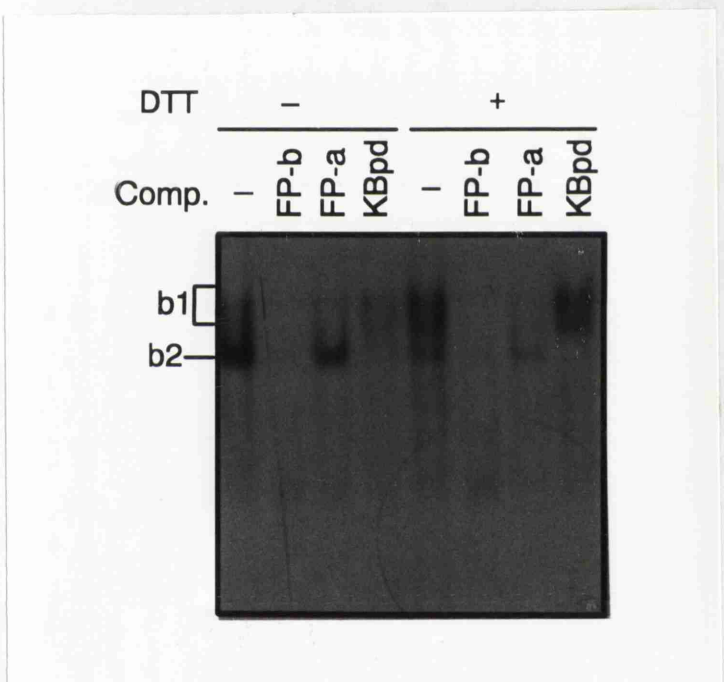
EMSA anslysis, in the presence of 5mM GTP, of nucleoprotein binding to MIP1α promoter site FP-b. 1 μg of nuclear extracts from 8 hr LPS stimulated RAW 264.7 cells was incubated with 0.1 ng of FP-b probe in the absence (-) or presence (+) of fresh 1 mM DTT, and in the absence (-) or presence (+) of 2.5 ng of FP-b , κBpd or FP-a competitor (Comp.). Specific retarded nucleoprotein complexes b1 and b2 are labelled. Two complexes with the characteristics (in terms of competition) of complex b1 are formed (see text) and are bracketed in the figure. Uncomplexed excess FP-b probe has migrated off the bottom of the gel.

Coding-strand sequences of oligonucleotides:

FP-a: CCCAGATTCCATTTCCTCATCTGCTAGGG

κBpd: CAACGGCAGGGGAATTCCCCTCTCCTT (Ballard *et al*, 1990)

FP-b: CTTAAAATTTTCCCTCCTCAC



12.1 OVERVIEW.

In this study, two sets of functional RAW 264.7 cell transient transfection data are presented, namely that shown in figure **10.3** (henceforth termed "Experiment 1") and that shown in figure **13.3** (henceforth termed "Experiment 2"). The difference between Experiment 1 and Experiment 2 is that the pM-350 promoter is 2-fold more active than pM-190 in Experiment 2, while in Experiment 1, both promoters have the same activity. Although the data in Experiment 1 is the mean of a greater number of transfections than that in Experiment 2, the difference between duplicate plates in individual experiments was minimal, suggesting that rather than experiment 1 being more accurate, a difference in transfection condintions between Experiments 1 and 2 may explain the different results.

In both Experiments 1 and 2, RAW CM was cell growth medium "conditioned" by RAW 264.7 cells for a defined period of time. However, in Experiment 1, the medium was obtained from RAW 264.7 cells after 2-3 days' incubation, whereas in Experiment 2, the medium was obtained from cells incubated for *at least* 3 days. A second difference was in the transient transfection procedure itself. In this procedure, the RAW 264.7 cells were transfected in OPTIMEM serum-free medium, then incubated for a defined period in fresh cell growth medium containing fresh 10% (v/v) FCS prior to the hGH assay period; however, in Experiment 1, this defined period was 36 hours, whereas in Experiment 2 it was 48 hours. The combined effects of these two differences would be that in Experiment 2, the positive regulatory effects of serum growth factors on the basal expression of the MIP1α promoter in the RAW 264.7 cells would be reduced.

This suggests that the MIP1α promoter region between -160 bp and -190 bp relative to the cap site (the FP-e region) contains a constitutive positively-acting promoter element that is serum-responsive, while the region between -190 bp and -350 bp contains a constitutive positively-acting promoter element that is not serum-responsive. Within the -190 bp to -350 bp region, only the FP-f site (-190 bp to -220

bp) is protected in *in-vitro* DNase1 footprinting experiments, suggesting that it is the FP-f site that contains the described constitutive positively-acting promoter element.

In order to further investigate nucleoprotein binding to these sites, overlapping double-stranded oligonucleotides FP-e and FP-f (see figure **11.1c**) were used in EMSA studies with nuclear extracts from unstimulated- and LPS-stimulated RAW 264.7 cells, and from control STO- and Swiss 3T3 fibroblast cells. The reason for using 3T3 fibroblast cells in these studies was that an AP-1-like nucleoprotein complex forms in EMSAs using FP-f probe (see below) and AP-1 proteins in 3T3 cells have been well-studied (Gillespie, 1991).

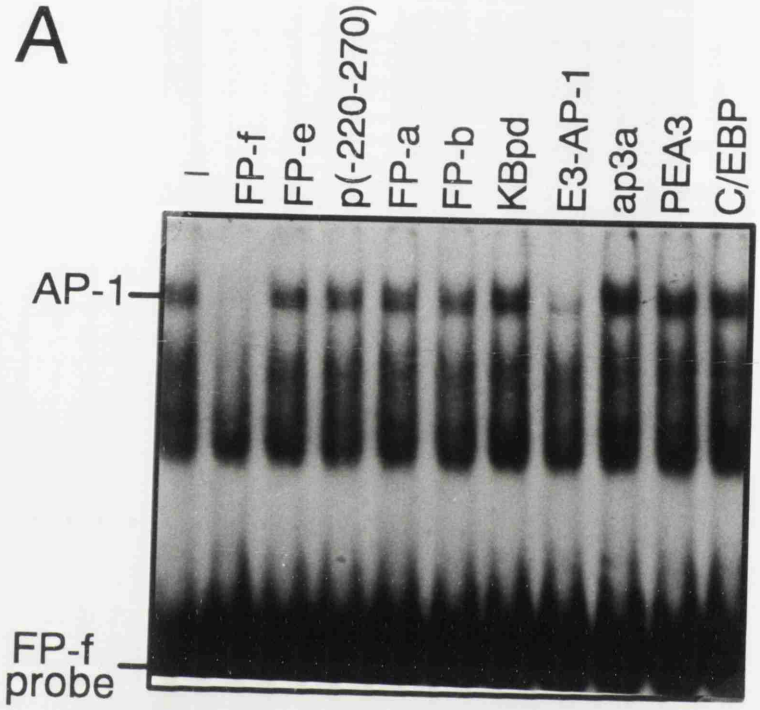
12.2 EMSA STUDIES OF NUCLEOPROTEIN BINDING TO THE MIP1α GENE DISTAL REGULATORY ELEMENT.

12.2.1 FP-f probe binds an AP-1-like nucleoprotein complex.

When FP-f probe is used in EMSAs, a slow-migrating complex is formed with nuclear extracts from unstimulated RAW 264.7 cells (figure **12.1a**). Slightly less of this complex is formed with nuclear extracts from LPS-stimulated- compared to unstimulated- RAW 264.7 cells (figures **12.2** and **12.4**). Of the competitor oligonucleotides used in the experiment shown in figure **12.1**, namely FP-a, FP-b, κBpd, αp3a, PEA3, C/EBP, FP-f and E3-AP-1, only FP-f and E3-AP-1 competitors competed out the slow-migrating complex (figures **12.1-12.4**). E3-AP-1 oligonucleotide contains a defined AP-1 transcription factor binding site (Frame *et al*, 1991), suggesting that FP-f might bind AP-1.

To further define the optimal conditions for binding of the slow-migrating complex to FP-f, conditions in EMSAs using FP-f probe were varied. In low salt conditions (70mM NaCl), binding of the FP-f AP-1-like complex was greatly increased in the presence of 1mM DTT (figure **12.2**), a REDOX-sensitivity which is characteristic of AP-1 DNA binding (Frame *et al*, 1991). Interestingly, binding of this complex to FP-f was also greatly enhanced by increasing the binding buffer salt concentration (150mM, figure **12.2**), though the presence of high salt *and* 1mM DTT did not allow optimal DNA binding (figure **12.2**). Further EMSAs with FP-f probe

A



B

Homology of FP-f oligo to
known AP-1 binding sites

AP-1 core	TGACTCA
E3-AP-1	TGACTAA
hTGFβ(a)	TGAGACGA
hTGFβ(b)	TGAGACTT
FP-f	TGAGACTT

Figure 12.1: AP-1-like nucleoprotein binding to the distal MIP1α gene promoter in EMSAs.

A: 1 μg of nuclear protein from unstimulated RAW 264.7 cells was incubated in EMSAs with 1 ng of MIP1α promoter FP-f probe in the presence or absence of 50 ng of competitor, as shown. The AP-1-like complex and the uncomplexed excess FP-f probe are labelled, and non-specific and/or variably-formed faster-migrating complexes are visible but not labelled. **B:** Homology between the MIP1α promoter FP-f oligonucleotide and known AP-1 binding sequences (see text). h, human; a and b, distal and proximal AP-1 binding sites of the human TGFβ promoter.

Coding strand sequences of competitors used:

FP-a: CCCAGATTCCATTTCCTCATCTGCTAGGG

C/EBP: GCTGCAGATTGCGCAATCTGCAGC (Landschultz *et al*, 1988)

κBpd: CAACGGCAGGGGAATTCCCCTCTCCTT (Ballard *et al*, 1990)

FP-b: CTTAAAATTTTCCCTCCTCAC

αP3a: GATCCAAACCAGCCAATGAGAACTGCTCCA (MacLeod *et al*, 1991)

FP-e: GACTTATATCTCAGAGATGCTATTCTTAGATATCCTGGG

FP-f: AAGAAGAAAGAATAGGTCACGAGTTGAGAGCTGAGACTTATAT

p(-220-270): TACCTCCTCCTGCTCATAAGAGAAACTACTTCCCC
ACAAGAAGAAAGAAT

E3-AP-1: CCGAAGTTCAGATGACTAACTCAGGG (Hurst *et al*, 1987)

PEA3: GATCCTCGAACTTCCTGCTCGAA (Xin *et al*, 1992)

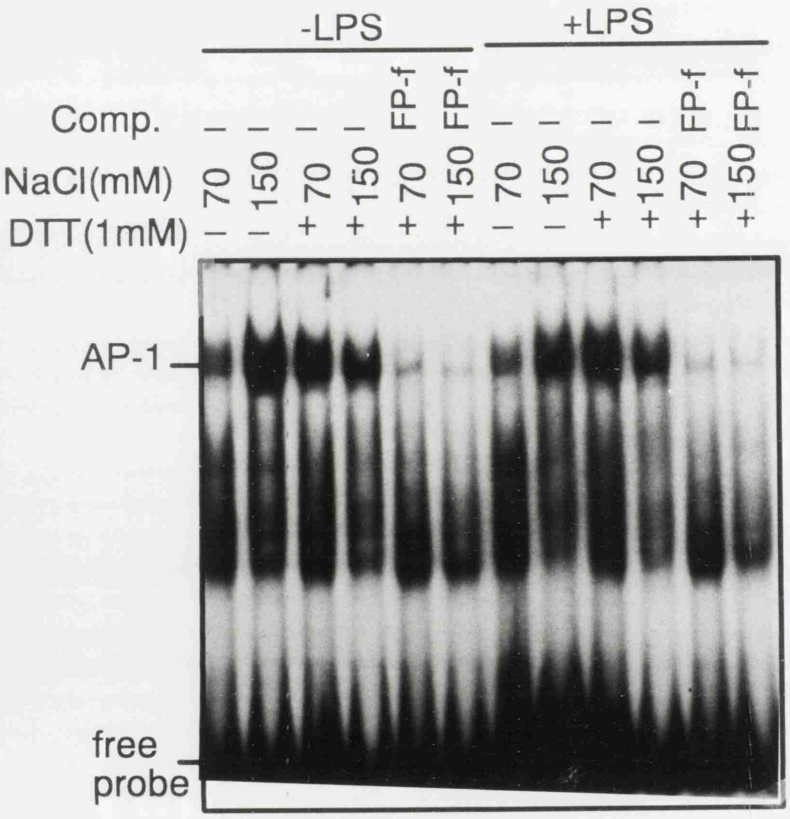

-LPS
+LPS
Comp.
NaCl(mM)
DTT(1mM)
– 70
– 150
+ 70
+150
+ 70
+150
– 70
– 150
+ 70
+150
+ 70
+150
–
–
–
–
FP-f
FP-f
–
–
–
–
FP-f
FP-f
AP-1
free
probe

Figure 12.2: REDOX- and salt-concentration sensitivity of AP-1-like nucleoprotein binding to the distal MIP1α gene promoter in EMSAs.

EMSAs in which 1 ng of FP-f probe was incubated with 1 μg of nuclear extract from unstimulated (-LPS) or 22 hour LPS-stimulated (+LPS) RAW 264.7 cells, in the absence (-) or presence (+) of 25 ng of FP-f competitor (Comp.), in the absence (-) or presence (+) of fresh 1 mM DTT, and at either low- (70 mM) or high (150 mM) sodium chloride (NaCl) concentration, as shown. The slow-migrating AP-1-like nucleoprotein complex (AP-1) and uncomplexed excess FP-f probe (free probe) are labelled. Rapidly-migrating non-specific- and/or variably present/competed nucleoprotein complexes are visible but not labelled.
Coding strand sequence of oligonucleotide used:
FP-f: AAGAAGAAAGAATAGGTCACGAGTTGAGAGCTGAGACTTATAT.

were therefore carried out in buffer containing 70mM NaCl and 1mM DTT. Further evidence for AP-1 binding to FP-f is shown in figure **12.3**, lanes 1-6, wherein an equivalently-migrating complex is formed with FP-f and E3-AP-1 probes, which cross-compete for binding.

As c-Fos is a component protein of AP-1 complexes (under certain cell growth conditions), and an anti-murine c-Fos antibody was available in the laboratory (Frame *et al*, 1991), I investigated whether the antibody could super-shift the AP-1-like RAW 264.7 cell nucleoprotein complex formed in EMSAs with either E3-AP-1 or FP-f probe; it could not (figure **12.3**, lanes 7-10). Although the presented data does not include a positive control, experiments were conducted in the same conditions as those successfully used in such experiments at the Beatson Institute with the same antibody (Frame *et al*, 1991).

It has recently been demonstrated that in NIH 3T3 fibroblast cells, different Fos family proteins are present in AP-1 complexes formed under different growth conditions (Kovary *et al*, 1992). Thus, in asynchronously-growing NIH 3T3 cells, c-Fos and FosB are expressed at very low levels, and Fra-1 and Fra-2 are the main Fos proteins complexed with c-Jun, while only in the first hour after serum stimulation does AP-1 in these cells consists mainly of c-Fos and c-Jun proteins (Kovary *et al*, 1992). By analogy, the lack of detectable c-Fos in the slow-migrating AP-1-like complex formed with FP-f probe and unstimulated RAW 264.7 nuclear extracts does not preclude that the complex is AP-1.

To investigate the possibility that the RAW 264.7 cell nucleoprotein complex formed in EMSAs with E3-AP-1 probe was distinct from AP-1, I investigated whether formation of the observed complex was dependent on the defined AP-1 binding site in the E3-AP-1 probe. In order to do this, mAP-1, an E3-AP-1 oligonucleotide specifically mutated at the AP-1 binding site (Hurst *et al*, 1987) (figure **12.4a**) was used as a competitor in EMSAs. When nuclear extracts from unstimulated- or LPS stimulated RAW 264.7 cells, or from serum-stimulated (4 hours) Swiss 3T3 cells are used in EMSAs with FP-f probe, an equivalently-migrating AP-1-like complex is formed, and this complex is not competed by mAP-1

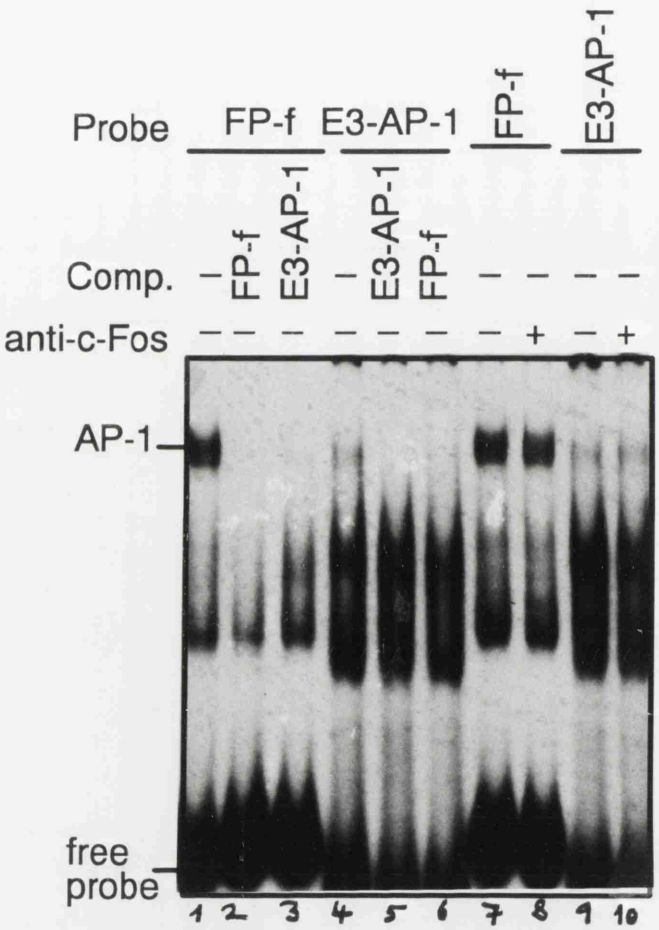

Probe
FP-f
E3-AP-1
FP-f
E3-AP-1
Comp.
–
FP-f
E3-AP-1
–
E3-AP-1
FP-f
–
–
–
–
anti-c-Fos
–
–
–
–
–
–
–
+
–
+
AP-1
free
probe
1
2
3
4
5
6
7
8
9
10

Figure 12.3: Similar nucleoprotein complexes formed in EMSAs with E3-AP-1 and MIP1α gene promoter FP-f probes.

EMSAs in which 1 ng of either FP-f probe or E3-AP-1 probe was incubated with 1 μg of nuclear extract from unstimulated RAW 264.7 cells, in the absence (-) or presence (+) of 50 ng of FP-f or E3-AP-1 competitor (Comp.). Anti-c-Fos protein antibody (anti-c-Fos) or control pre-immune serum (Frame *et al*, 1991) were present/absent in the EMSA incubations as follows: Lanes 1-6, no pre-immune and no anti-c-Fos (-); lanes 7 and 9, pre-immune serum present, anti-c-Fos protein antibody absent (-); lanes 8 and 10, pre-immune serum absent, anti-c-Fos protein antibody present (+). The slow-migrating AP-1-like nucleoprotein complex (AP-1) formed with FP-f and E3-AP-1 probes, and uncomplexed excess FP-f and E3-AP-1 probes (free probe) are labelled. Rapidly-migrating non-specific- and/or variably present/competed nucleoprotein complexes are visible but not labelled.
Coding strand sequences of oligonucleotides used:
FP-f: AAGAAGAAAGAATAGGTCACGAGTTGAGAGCTGAGACTTATAT.
E3-AP-1: CCGAAGTTCAGATGACTAACTCAGGG (Hurst *et al*, 1987)

Figure 12.4: Investigation of the site of binding of specific nucleoprotein complexes to E3-AP-1 and MIP1α gene promoter FP-f probes in EMSAs.

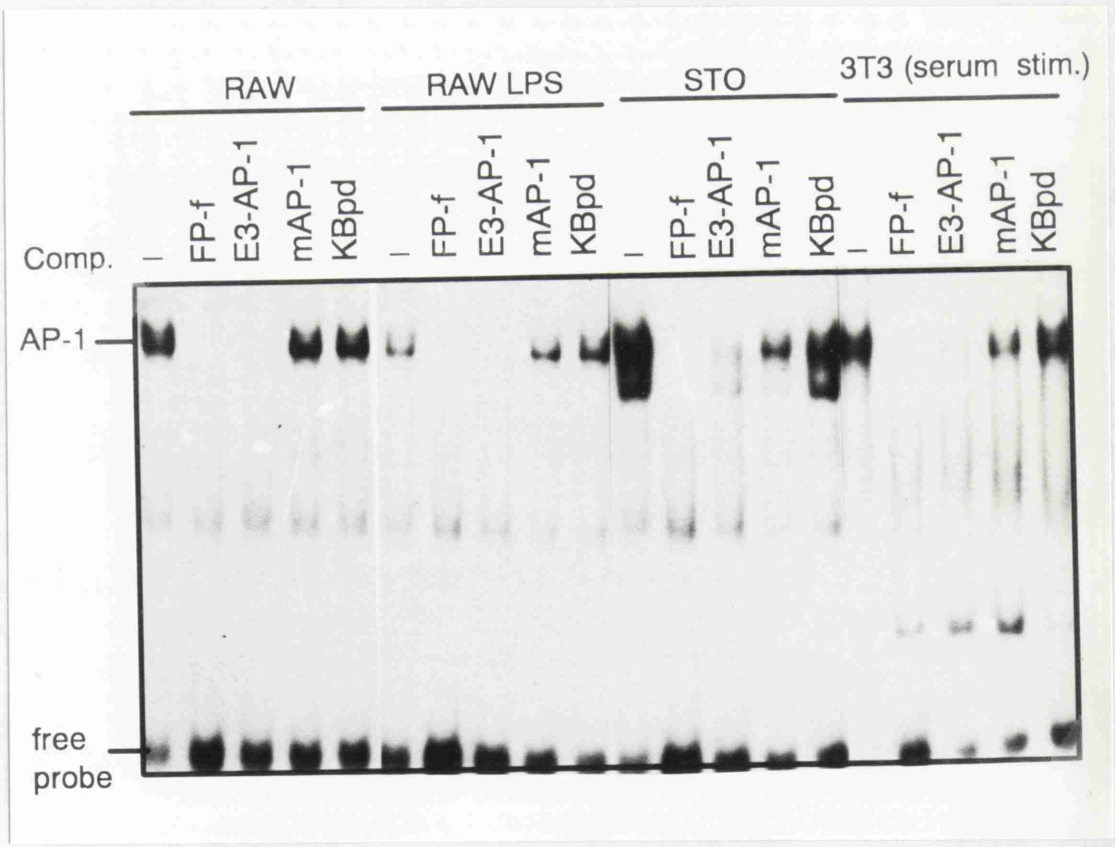
A: Sequence alignment of the AP-1 protein DNA binding site of the E3-AP-1 oligonucleotide and the corresponding sequence of the mutated E3-AP-1 oligonucleotide, mAP1. Upper-case letters and lower-case letters in the latter sequence denote identity- and non-identity, respectively, with the corresponding nuleotide in the E3-AP-1 sequence. **B:** EMSAs in which 1 μg of nuclear extract from either unstimulated RAW 264.7 cells (RAW), 22 hr LPS-stimulated RAW 264.7 cells (RAW LPS), STO cells (STO) or 4 hr fresh 10% (v/v) FCS-stimulated Swiss 3T3 cells (3T3 (serum stim.)), was incubated with 1 ng of FP-f probe, in the absence (-) or presence (+) of 50 ng of FP-f, E3-AP-1, mAP-1 or κBpd competitor (Comp.). The slow-migrating AP-1-like nucleoprotein complex (AP-1) formed with FP-f probe, and uncomplexed excess FP-f probe (free probe) are labelled. A specific nucleoprotein complex formed with FP-f probe and STO cell nuclear extracts, that migrates slightly more rapidly than the AP-1 complex (see text), plus rapidly-migrating non-specific- and/or variably present/competed nucleoprotein complexes, are visible but not labelled.

Coding strand sequences of oligonucleotides used:
FP-f: AAGAAGAAAGAATAGGTCACGAGTTGAGAGCTGAGACTTATAT.
E3-AP-1: CCGAAGTTCAGATGACTAACTCAGGG (Hurst *et al*, 1987)
mAP-1: CCGAAGTTCAGATTCAGAACTCAGGG
κBpd: CAACGGCAGGGGAATTCCCCTCTCCTT (Ballard *et al*, 1990)

A

E3-AP-1	**CCGAAGTTCAGATGACTAACTCAGGG**
AP-1	**TGACTAA**
mutant AP-1	**TtcagAA**
mAP-1	**CCGAAGTTCAGATtcagAACTCAGGG**

B



competitor (figure **12.4b**). In the same experiment using STO nuclear extracts, two specific compelexes were formed with FP-f probe, both of which were competed by both FP-f and E3-AP-1 competitors (figure **12.4b**). However, mAP-1 competitor specifically competed for the faster-migrating complex, but not for the AP-1-like complex (figure **12.4b**), confirming that the slow-migrating AP-1-like complex formed with FP-f and E3-AP-1 probes specifically forms on an AP-1 binding site. Furthermore, the formation of the faster-migrating E3-AP-1-competed complex with FP-f probe and STO nuclear extracts shows that in addition to AP-1, probes FP-f and E3-AP-1 can both bind the same additional complex that is not related to AP-1.

Comparison of the sequence of FP-f probe with that of known AP-1 DNA binding sites (figure **12.1b**) reveals little homology to the E3-AP-1 oligo AP-1 binding site or to a canonical AP-1 binding site (Frame *et al*, 1991; Gillespie, 1991). However, two sites within the second promoter of the human TGF-β1 gene, are functionally responsive to TGF-β1- and phorbol ester, and this functional responsiveness is conferred by the binding of AP-1 (Kim *et al*, 1989; Kim *et al*, 1990), and one of these sites shows close homology to a site within the FP-f oligonuleotide, while the other is identical to this FP-f site (figure **12.1b**).

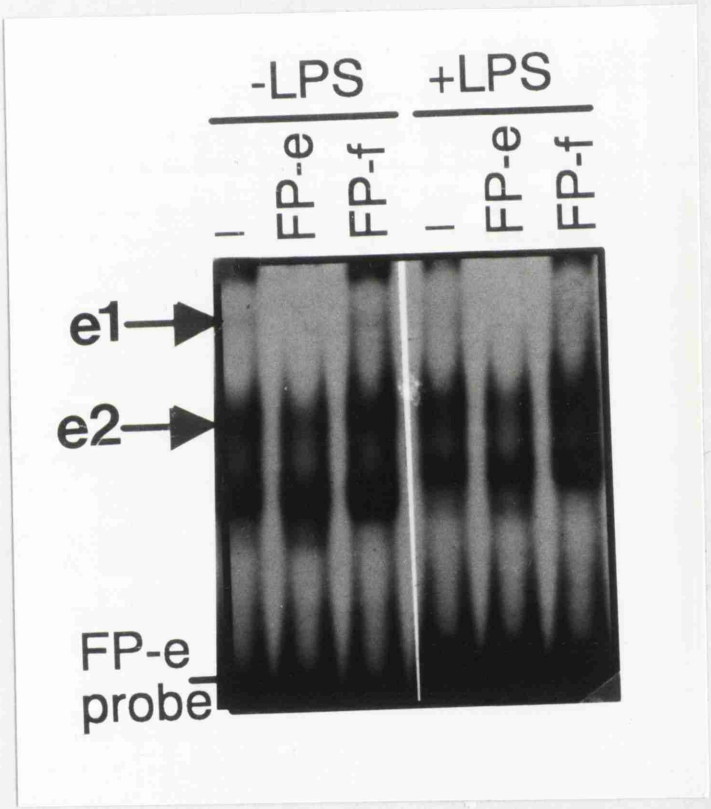
12.2.2 Specific RAW 264.7 cell nucleoprotein binding to FP-e probe.

Low levels of a specific slow-migrating nucleoprotein complex (e1) are formed using FP-e probe with unstimulated and LPS stimulated RAW 264.7 cell nuclear extracts, and the intensity of this complex decreases upon LPS stimulation (figure **12.5**). A more intense faster-migrating nucleoprotein complex (e2) that is partially competed by 25-fold molar excess of FP-e but not FP-f competitor is also obtained (figure **12.5**). Because this complex is only partially competed by FP-e competitor, it may not be specific, and has not been further investigated.

Two possible explanations for the only low levels of complex e1 formed with FP-e probe in the above EMSAs could be that the protein(s) forming complex e1 are in low abundance in RAW 264.7 cells, or that the EMSA binding conditions did not favour formation of complex e1. To investigate the latter possibility, a range of DNA binding conditions (for example, variation of salt concentration, REDOX conditions,

Figure 12.5: EMSA analysis of RAW 264.7 cell nucleoprotein binding to MIP1α promoter site FP-e.

EMSAs, in which 1 μg of nuclear extract from either unstimulated- (-LPS) or 22 hour LPS-stimulated RAW 264.7 cells was incubated with 1 ng of FP-e probe, in the absence (-) or presence (+) of 25 ng of FP-e or FP-f competitor. Specific nucleoprotein complex e1, nucleoprotein complex e2, which may not be specific (see text), and uncomplexed excess FP-e probe are labelled. A rapidly-migrating non-specific nucleoprotein complex is visible but is not labelled.
Coding strand sequences of oligonucleotides used:
FP-e: AGACTTATATCTCAGAGATGCTATTCTTAGATATCCTGGG
FP-f: AAGAAGAAAGAATAGGTCACGAGTTGAGAGCTGAGACTTATAT.



etc.) were used in further EMSAs with FP-e probe. However, no significant alteration in complex formation was achieved, so this data will not be presented. This could mean that footprint FP-e (figure **11.1**) is due to non-specific nucleoprotein binding, though this is unlikely because the MIP1α promoter region encompassed by FP-e is functionally important in transfection studies (figure **10.3**).

12.2.3 Is RAW 264.7 cell nucleoprotein binding to MIP1α promoter footprinted sites FP-e and FP-f co-operative ?

In addition to the low levels of specific nucleoprotein binding to FP-e in EMSAs, it is evident that the proposed AP-1 binding site within FP-f probe is actually situated slightly downstream of the FP-f site- and overlapping (but not within) the FP-e site-, obtained in *in-vitro* DNase1 footprinting studies with RAW 264.7 cell nuclear extracts (figure **11.1c**). Hence, no specific RAW 264.7 cell nucleoprotein binding to the footprinted FP-f sequences-, and possibly only low levels of specific RAW 264.7 cell nucleoprotein binding to the footprinted FP-e sequences-, was detected in EMSAs. However, the FP-f footprinted site contains a conserved cytokine promoter CK-2 motif (Discussion figure **16.2**; Nimer *et al*, 1988), and the FP-e site contains an inverted repeat motif, indicative of a potential dimeric transcription factor binding site (see chapter **2**).

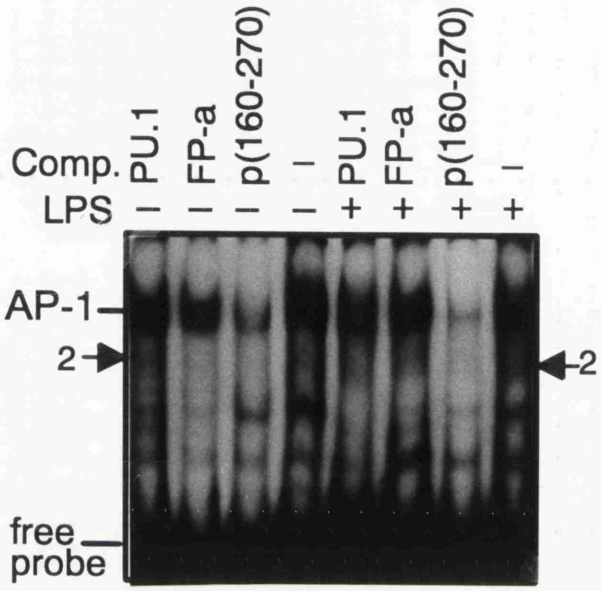
As RAW 264.7 cell nucleoprotein binding to the FP-e and FP-f sites is detectable in *in-vitro* footprinting experiments, the above observations raised the possibility that RAW 264.7 cell nucleoprotein binding to FP-e and FP-f is co-operative. To investigate this, the MIP1α promoter HincII and EcoRV restriction enzyme recognition sites that border the FP-e-FP-f region were used to generate probe p(160-270) (figure **12.6b**) which was used in EMSAs with unstimulated and LPS-stimulated RAW 264.7 cell nuclear extracts (figure **12.6a**).

As shown in figure **12.6a**, preliminary EMSA analysis with this probe gives an abundant and specific slow-migrating nucleoprotein complex that is slightly reduced upon LPS stimulation (figure **12.6a**). Though not tested, this complex is probably AP-1, as obtained with FP-f probe (figures **12.1-12.4**). In addition, a faster migrating

Figure 12.6: Investigation of co-operative binding of nucleoproteins to MIP1α promoter footprinted sites FP-e and FP-f in EMSAs.

A: Sequence of probe p(160-270), which contains all of the MIP1α promoter footprint FP-f sequence and 30/34 bps of the footprint FP-e sequence. The sequence of the MIP1α promoter between -283 bp and -120 bp. Footprinted sequences FP-f, FP-e, FP-d and the 5' part of dFP-c are in bold typeface. The sequences encompassed by oligonucleotides FP-f, FP-e and FP-d, and the 5' end of the sequence encompassed by oligonucleotide dFP-c, are denoted by unbroken lines above the promoter sequence. The proposed C/EBP-family binding sequence in FP-d, and the 5' end of the proposed C/EBP family binding sequence in dFP-c, are denoted by dashed lines above the respective sequences. The HincII and EcoRV restriction enzyme recognition sites, which are at the 5' and 3' ends of the p(160-270) sequence, respectively, are labelled. Sequence identity between the MIP1α promoter and the corresponding region of the human MIP1α gene LD78α promoter (LD78) is denoted by vertical lines between the two sequences. **B**: EMSAs, in which 1 ng of p(160-270) probe was incubated with 1 μg of nuclear extract from unstimulated (LPS -) or 22 hr LPS-stimulated (LPS +) RAW 264.7 cells, in the absence (-) or presence (+) of 25 ng of PU.1, FP-a or p(160-270) competitor. The slow-migrating AP-1-like nucleoprotein complex, the specific complex 2, and uncomplexed excess p(160-270) probe (free probe) are labelled. Further rapidly-migrating nucleoprotein complexes, which may or may not be specific, are visible but not labelled.
Coding strand sequences of oligonucleotides used:
FP-a: CCCAGATTCCATTTCCTCATCTGCTAGGG
PU.1: ATAACCTCTGAAAGAGGAACTTGGTTAGGT (Klemsz *et al*, 1990)
p(160-270): see figure.

A



B

5' end of p(160-270) probe

```
      -283                HincII
MIP1α AAGAATAGCCCTGGGTTGACACTGTCCTACCTCCTCCTGCTCATAAGAGAAACTACT
      |||||||||| |||||||||| ||  |||| ||  |||| | |||||||| | |
LD78  AAGAATAGCCTTGGGTTGACATATACC--CCTCTTCACACTCACAGGAGAAACCATT
```

```
                                                  _____FP-e_____
                  _____________________FP-f_____________________________
      -231
MIP1α TCCCCACAAGAAGAAAGAATAGGTCACGAGTTGAGAGCTGAGACTTA-----TATCT
      |||| |   |||   | || | |||| ||||||||||||||||| |||      || ||
LD78  TCCCTAT--GAAACTATAACAAGTCATGAGTTGAGAGCTGAGAGTTAGAGAATAGCT
```

```
      ___________FP-e_____________                  ______dFP-c
                                  ________FP-d_____________
      -175                        ----------          ----
MIP1α CAGAGATGCTATTCTTAGATATCCTGGGCCCCTGTGGTCACTGTGGACCCTGGGTTG
      || ||||||||||||| ||||||||| ||||||||||||||   |||||||| ||||
LD78  CAAAGATGCTATTCTTGGATATCCTGAGCCCCTGTGGTCACCAGGGACCCTGAGTTG
```

EcoRV

3' end of p(160-270) probe.

complex (complex 2) specific for probe p(160-170) is obtained (figure **12.6a**), though this complex has yet to be investigated further.

It is also possible that nucleoprotein binding to the FP-e-FP-f region is enhanced in the presence of sequences proximal to -160 bp. EMSA probes to investigate this possibility could be generated by PCR. Evidence for such co-operative binding to the MIP1α promoter will be presented below, and further addressed in the Discussion section.

CHAPTER 13: FUNCTIONAL ANALYSIS OF THE MIP1α GENE PROXIMAL AND DISTAL PROMOTER REGIONS, USING PCR SITE-DIRECTED MUTAGENESIS.

13.1 OVERVIEW.

The presented functional data shows that the proximal MIP1α gene promoter region contains constitutive macrophage-specific and LPS- and serum-responsive promoter elements. The *in-vitro* MIP1α promoter nucleoprotein binding analysis described in chapter **11** reveals a complex pattern of binding to the pM-160 MIP1α gene proximal promoter sequences. This region contains five footprinted sites, FP-a, FP-b, pFP-c, dFP-c and FP-d, all of which bind RAW 264.7 cell nucleoprotein complexes *in-vitro* in a constitutive and LPS-inducible manner, and at least four of which also bind RAW 264.7 cell nucleoproteins in a serum-responsive manner.

Observed nucleoprotein binding to FP-b appears to be restricted to Rel family members, while that to pFP-c, dFP-c and FP-d is restricted to C/EBP family members in RAW 264.7 cells (though FP-d binds other, unidentified proteins present in STO and MEL cell nuclear extracts). However, the presented evidence suggests that FP-a can bind C/EBP-, Rel- and Ets- (PU.1 and Spi-B) family proteins. In addition, the FP-a sequence shows homology to murine G-CSF gene promoter element 1 (mGPE-1), as both contain a C/EBP family binding site and a "CK-1" motif, which is present in several cytokine promoters (Nishizawa *et al*, 1990). Both the C/EBP family binding

site and the CK-1 motif are required for LPS-inducibility of the murine G-CSF promoter in macrophage cell-lines, although observed macrophage cell-line nucleoprotein binding to FP-a and mGPE-1 in EMSAs is only to the C/EBP family binding site (Nishizawa *et al*, 1990; figures **11.2b** and **11.4a**). FP-a also contains a consensus interferon (IFN) α/β responsive element (ISRE), and the ISRE of the PF4 family IP-10 gene is functionally responsive to both IFNα/β and LPS in RAW 264.7 cells (Ohmori *et al*, 1993), suggesting that the FP-a ISRE may contribute to MIP1α promoter LPS- and IFNα/β-responsiveness in RAW 264.7 cells. The various potential and observed nucleoprotein binding sites of FP-a, and their overlapping nature, are summarised in figure **13.1**.

The *in-vitro* analysis of nucleoprotein binding to the distal regulatory element of the MIP1α promoter suggests that a low-abundance RAW 264.7 cell nucleoprotein complex can bind to the FP-e sequence, and that an AP-1-like nucleoprotein complex can bind to a DNA site situated between the FP-e and FP-f footprinted regions. Functional- and footprinting data suggests that the FP-e region contains a constitutive positively-acting promoter element that is serum-responsive, while the FP-f region contains a constitutive positively-acting promoter element that is not serum-responsive.

13.2 SYNTHESIS OF MIP1α POINT-MUTATED PROMOTERS.

In order to address the functional contribution of the described MIP1α promoter sites, different point mutations were introduced into this region in such a way as to (hopefully) affect the binding of distinct subsets of proteins capable of binding to the different footprinted sites. The mutations introduced, and the nucleoprotein binding that each mutation should affect is summarized in figure **13.1**. *In-vitro* binding of C/EBP family members to site FP-d (as measured by EMSAs, figures **11.4a** and **11.4b**) is weaker than to FP-a, pFP-c and dFP-c, correlating with the lower C/EBP binding site homology of FP-d (figure **11.2a**). Hence, mutants dm1 to dm4, spanning FP-d, were synthesised (figure **13.1**), in order to ensure that the correct FP-d C/EBP family binding site was mutated. Because the exact site of *in-vitro* nucleoprotein

MIP1α promoter : transcription factor binding sites and mutants

	CAGATTCCAT CK-1 CAGATTCCATTTCC ISGF3 TCCATTTCCT c-Rel TTCCTC PU.1, Spi-B TTTCCTCATC C/EBP		
Mutant		Knockout	Mutant promoter size
FP-a	CCCAGATTCCATTTCCTCATCTGCTAGGG	–	
FP-am40	CCCAGAaTtCATgaatTCATCTGCTAGGG	All	350 bp
FP-am41	CCCgaATTCCATTTCCTCtTtTGCTAGGG	C/EBP, ISGF3, CK-1	160 bp 350 bp
FP-am44	CCCgaATTCCAaTTaCaCAaCTGCTAGGG	PU.1, Spi-B, ISGF3, c-Rel, CK-1	500 bp
FP-am46	CCCAGATTCCATTTCCaCATCTGCTcgaG	PU.1, Spi-B	350 bp
FP-am47	CCCAGATTCCATTTCCTCtTtTGCTcGaG	C/EBP	350 bp
FP-am48	CCCgagcTCCATTTCCTCATCTGCTAGGG	ISGF3, CK-1	350 bp
	AATTTTCC NF-KB, c-Rel		
FP-b	CTTAAAATTTTCCCTCCTCAC	–	
FP-bm30	CTTAAAATgagCtCTCCTCAC	NF-KB, c-Rel	350 bp
FP-bm35	CTTAAAATTcTagaTCtagAC	NF-KB, c-Rel	350 bp, 500 bp
	TCATGACACCAT C/EBP		
pFP-c	TCCATCATGACACCATTGCTGTG	–	
pFP-cm10	TCCATCATGACACgAaTtCTGTG	C/EBP	350 bp
	GTTGTGTAAT C/EBP		
dFP-c	CCCTGGGTTGTGTAATATCCA	–	
dFP-cm20	CCCTGaGcTcTGTgAgcTCCA	C/EBP	350 bp
	CCTGTGGTCA C/EBP		
FP-d	GGGCCCCTGTGGTCACTGTGGACCCTG	–	
FP-dm1	GaattCtTGTGGTCACTGTGGACCCTG	?	350bp
FP-dm2	GGGCCCgaaTtcTCACTGTGGACCCTG	C/EBP	350bp
FP-dm3	GGGCCCCTGTGagaAtTcTGGACCCTG	?	350bp
FP-dm4	GGGCCCCTGTGGTCACTagaattCCTG	–	350bp
FP-e	AGACTTATATCTCAGAGATGCTATTCTTAGATATCCTGGG		
FP-em123	gaattcATATCTCAGAGATGCTATTCTTAGATATCCTGGG		190 bp
FP-em223	AGACTTgaattcCAGAGATGCTATTCTTAGATATCCTGGG		190 bp
FP-em323	AGACTTATATCTgaattcTGCTATTCTTAGATATCCTGGG		190 bp 350 bp
FP-em423	AGACTTATATCTCAGAGAgaattcTCTTAGATATCCTGGG		190 bp 350 bp
FP-em523	AGACTTATATCTCAGAGATGCTATgaattcATATCCTGGG		190 bp

Figure 13.1: MIP1α promoter footprinted sequences: mutational analysis of transcription factor binding sites.

Wild-type and mutant sequences of MIP1α promoter footprinted sites: FP-a (wild-type) and FP-am41, FP-am44, FP-am46, FP-am47, FP-am48 (mutants); FP-b (wild-type) and FP-bm30, FP-bm35 (mutants); pFP-c (wild-type) and pFP-cm10 (mutant); dFP-c (wild-type) and dFP-cm20 (mutant); FP-d (wild-type) and FP-dm1, FP-dm2, FP-dm3, FP-dm4 (mutants); FP-e (wild-type) and FP-em1, FP-em2, FP-em3, FP-em4, FP-em5 (mutants). Potential transcription factor DNA binding sites, identified in this study or by sequence comparison (see text), are aligned above the relevant wild-type footprinted sequence (eg. FP-a), and the name of the aligned sequence (eg. "CK-1") or the transcription factors that potentially bind to the aligned sequence (eg. "ISGF3") are shown. Mutated forms of the wild-type footprinted sequences are aligned below each wild-type sequence, the point mutations in these mutants are denoted by lower-case sequence, and the sequence homology or potential transciption factor binding site(s) affected by such mutations are listed to the right of each mutant sequence, in the column headed "Knockout". In cases where mutations are not expected to- or may not disrupt transcription factor binding, "-" or "?" is listed in the Knockout column for that mutant. All point mutations, except for FP-am46 and FP-am47, incorporate a restriction enzyme recognition site into the footprinted sequence (see figure). The listed point mutations were incorporated into the sequence of MIP1α promoter constructs pM-160, pM-350 or pM-500 (mutant promoter size 160 bp, 350 bp or 500 bp, respectively), as shown, using PCR site-directed mutagenesis, and the mutant promoters were cloned into the promoterless p0GH hGH gene-containing plasmid.

binding to the FP-e site was unknown, several mutant promoters containing different point mutations across the whole of FP-e were synthesised (figure **13.1**). The FP-f footprinted region of the distal regulatory element has so far not been mutated.

The mutations listed in figure **13.1** were introduced into the MIPα promoter by PCR site-directed mutagenesis, and the mutant promoters subcloned into the HindIII (5') and BamH1 (3') sites immediately upstream of the hGH gene in the p0GH vector. Each mutant promoter-containing plasmid, named after the mutation contained in that promoter, is listed in Table **6.1**, along with the names of the PCR primers used to generate each mutant. The sequences of the PCR primers are shown in Table **6.4**.

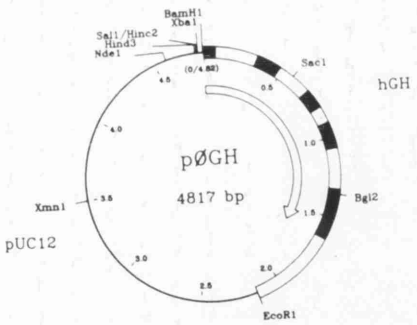
None of the mutant promoters listed in Table 1 has yet been sequenced, so that data presented below, in which some of these mutant promoters were tested in transient transfection assays in RAW 264.7 cells, are only preliminary. The point mutations introduced into MIP1α mutant promoters were in the form of restriction enzyme recognition sites, so that restriction enzyme digestion of mutant MIP1α promoter-containing plasmid constructs could verify that a promoter DNA fragment of the correct size and containing the correct restriction enzyme recognition site was contained in each construct. However, in the absence of sequencing, it is not possible to exclude the possibility that other unwanted point mutations were introduced into the constructs in the PCR/ cloning procedures.

Figure **13.2** shows restriction enzyme analyses of the mutant MIP1α promoter plasmid constructs that have so far been tested in functional assays. Figure **13.2b** shows restriction analysis of plasmids containing 350 bp MIP1α mutant promoters, and each plasmid was digested with either EcoR1 or Sac1, depending on which of these restriction enzyme sites had been incorporated into each mutant promoter (figure **13.1**, and Table **6.2**). The p0GH plasmid contains a single Sac1 recognition site ~500 bp from-, and a single EcoR1 site ~2.2 kbp from-, the site of subcloning of mutant MIP1α promoters into the vector (figures **13.2a** and Table **6.2**). Hence, restriction fragments of the correct size were generated from all plasmids except for pGHM-350am46 and pGHM-350am47 (figure **13.2b**, lanes 8 and 10, respectively). The "Sac1" restriction enzyme recognition sites contained in these latter two mutant

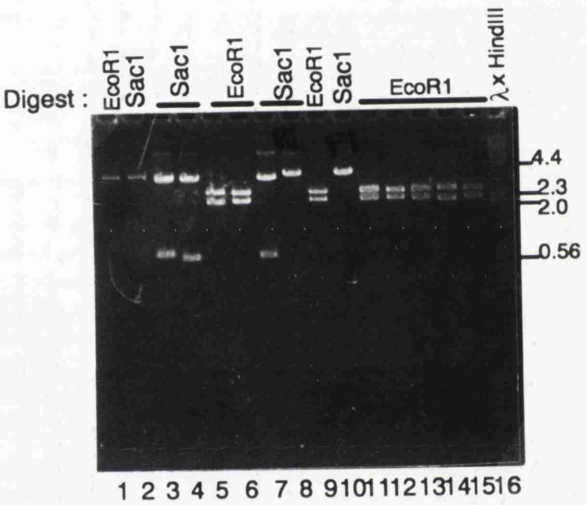
Figure 13.2: MIP1α gene mutant promter constructs: restriction enzyme analysis.

A: MIP1α mutant promoters were incorporated into the HindIII (5') and BamH1 (3') sites of the promoterless hGH gene-containing plasmid p0GH, which contains single recognition sites for Nde1, Sac1 (Sst1) and EcoR1 restriction enzymes at the positions indicated, and which were used in the analysis of mutant promoter constructs. **B:** Restriction enzyme digestion of MIP1α mutant promoter constructs in p0GH. Plasmids containing mutant promoter constructs were digested with either EcoR1 or Sac1 (Sst1) restriction enzyme (as shown), the digested DNA was purified, then electrophoresed on a 1.2% (w/v) agarose gel, next to molecular weight markers (λ phage digested with HindIII, lane 16: size (in kbps) of markers is shown to the right of the photograph), and p0GH plasmid digested with either EcoR1 (lane 1) or Sac1 (lane2), stained with ethidium bromide and photographed.
Lane 3, pGHM-350dcm20 (MIP1α dFP-cm20 mutant 350 bp promoter incorporated into p0GH) digested with Sac1
Lane 4, pGHM-350am48 digested with Sac1
Lane 5, pGHM-350pcm10 digested with EcoR1
Lane 6, pGHM-350dm2 digested with EcoR1
Lane 7, pGHM-350bm30 digested with Sac1
Lane 8, pGHM-350am47 digested with Sac1
Lane 9, pGHM-350em4 digested with EcoR1
Lane 10, pGHM-350am46 digested with Sac1
Lane 11, pGHM-350dm1 digested with EcoR1
Lane 12, pGHM-350em3 digested with EcoR1
Lane 13, pGHM-350dm3 digested with EcoR1
Lane 14, pGHM-350am40 digested with EcoR1
Lane 15, pGHM-350dm4 digested with EcoR1
The expected ~500 bp Sac1 restriction enzyme fragment is visible in lanes 3, 4 and 7, but not in lanes 8 and 10, as the Sac1 restriction enzyme "recognition sequence" in pM-350am47 and pM-350am46 is in inverted orientation. In lanes 5, 6, 9, 11, 12, 13, 14 and 15, the two expected ~2.3 kbp EcoR1 restriction fragments are visible.
C: Restriction enzyme digestion of MIP1α mutant promoter constructs in p0GH. Plasmids containing mutant promoter constructs were digested with either EcoR1 or Nde1, then resolved as in **(B)**, next to molecular weight markers (phage πX-174 digested with HaeIII, or λ phage digested with HindIII, as shown: size of markers (in kbps) is shown to the right of the photograph). Lanes contain the following digested plasmids:
Lane 1, pGHM-190em5 digested with EcoR1
Lane 2, pGHM-190em5 digested with Nde1
Lane 3, pGHM-190em4 digested with EcoR1
Lane 4, pGHM-190em4 digested with Nde1
Lane 5, pGHM-190em2 digested with EcoR1
Lane 6, pGHM-190em2 digested with Nde1
Lane 7, pGHM-190em1 digested with EcoR1
Lane 8, pGHM-190em1 digested with Nde1
Lane 9, pGHM-190em3 digested with EcoR1
Lane 10, pGHM-190em3 digested with Nde1
The two ~2.3 kbp EcoR1 restriction fragments expected in lanes 1, 3, 5, 7 and 9 are visible but migrate as a single band (if two sites were not present, a band of ~4.8 kbps would be obtained (see **A**)), and the single Nde1 restriction fragment expected in lanes 2, 4, 6, 8 and 10 is visible.

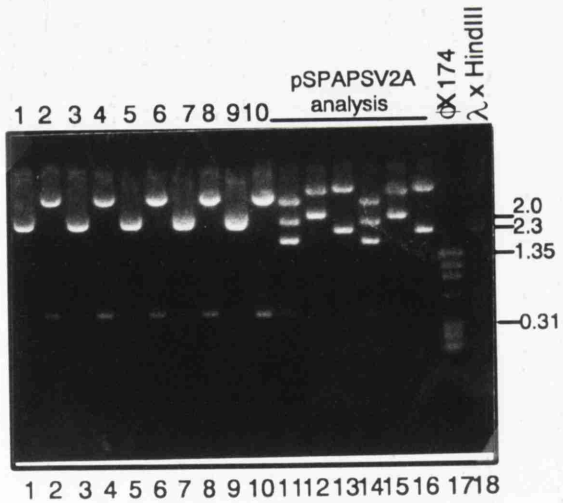
A


BamH1
Xba1
Sal1/Hinc2
Hind3
Nde1
Sac1
hGH
pØGH
4817 bp
Xmn1
Bgl2
pUC12
EcoR1

B


Digest :
EcoR1
Sac1
Sac1
EcoR1
Sac1
EcoR1
Sac1
EcoR1
λ x HindIII
4.4
2.3
2.0
0.56
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

C


1 2 3 4 5 6 7 8 9 10
pSPAPSV2A
analysis
ØX 174
λ x HindIII
2.0
2.3
1.35
0.31
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

promoters was accidentally designed and inserted in the wrong orientation (figure **13.1** and Table **6.4**), so that pGHM-350am46 and pGHM-350am47 may therefore actually contain the correct point mutations.

Figure **13.2c** shows restriction analysis of plasmids containing 190 bp MIP1α mutant promoters. As the p0GH plasmid and the 190 bp MIP1α promoter each contain a single Nde1 recognition site (figures **8.7** and **13.2a**), the size of the mutant MIP1α promoter inserts in the p0GH plasmid was verified by Nde1 digestion (figure **13.2c**, lanes 2, 4, 6, 8, 10) and the expected ~400 bp Nde1 restriction fragments were generated (figures **13.2a** and **13.2c**). Each of the 190 bp MIP1α mutant promoters incorporates an EcoR1 site at the site of point mutation (figure **13.1** and Table **6.4**), so that EcoR1 digestion of constructs not containing the correct mutation should generate an ~5 kbp DNA fragment (figure **13.2a**), while an EcoR1 fragment doublet of ~2.3 kbp should be generated from EcoR1 digestion of constructs containing the correct mutation, as is observed (figure **13.2c**, lanes 1, 3, 5, 7, 9).

Thus far, only the FP-d and FP-e mutant promoters have been functionally tested, in preliminary transient transfection assays in RAW 264.7 cells. Controls in these assays were wild-type pM-160, pM-190 and pM-350 promoters, as LPS responsiveness of the mutant promoters could then be compared to that of the LPS responsive pM-160 promoter, and mutant promoters are of either pM-190 or pM-350 length.

13.3 EFFECT OF FP-e POINT MUTATIONS ON pM-190 PROMOTER ACTIVITY IN RAW 264.7 CELLS.

13.3.1 Activity of pM-190 FP-e point-mutant promoters in unstimulated RAW 264.7 cells.

As shown in figures **10.3** and **13.3**, the MIP1α promoter FP-e site (-160 bp to -190 bp) contains a promoter element with strong constitutive activity in unstimulated RAW 264.7 cells. Mutant and wild-type MIP1α promoter constructs were transfected into RAW 264.7 cells (chapter **7**), then incubated for the test period in RAW CM. Mutant promoter pM-190em1 is 1.5-fold more active-, while pM-190em2, pM-

190em3, pM-190em4 and pM-190em5 are respectively 60%-, 3%, 3% and 10% as active as the wild-type pM-190 promoter in unstimulated RAW 264.7 cells (figure **13.3**). This suggests that a region of at least 24 bp (em2-em5) within FP-e is involved in the binding of positively-acting transcription factor(s). In contrast, the wild-type sequence mutated in mutation em1 (figure **13.1**), which contains the putative AP-1 binding site overlapping the 5' end of footprinted site FP-e (figure **11.1c**), may be involved in binding a negative regulatory transcription factor. Further evidence for this will be presented in the Discussion section, in light of further results presented below.

13.3.2 Activity of pM-190 FP-e point-mutant promoters in LPS stimulated RAW 264.7 cells.

Mutant and wild-type MIP1α promoter constructs were transfected into RAW 264.7 cells (chapter **7**), then incubated for the test period in RAW CM in the presence of 2 μg/ml of LPS. Mutant promoter pM-190em1 is induced only 1.5-fold-, and wild-type promoter pM-190 only 2.3-fold by LPS stimulation of RAW 264.7 cells, so that they have equivalent ***total activities*** after LPS stimulation (figure **13.3**), while mutant promoter pM-190em2 is induced only 2.2-fold, and has ~60% of the ***total activity*** of pM-190 after LPS stimulation (figure **13.3**). In contrast, although mutant promoters pM-190em3, pM-190em4 and pM-190em5 have very low unstimulated activity, they are are respectively induced 25-fold, 17-fold and 8-fold by LPS stimulation of RAW 264.7 cells, so that ***total activities*** of pM-190em3 and pM-190em5 are similar to-, and that of pM-190em4 is ~85%- of the total activity of the wild-type pM-160 promoter after LPS stimulation (figure **13.3**). Possible mechanisms for these results will be adressed in the Discussion section.

13.4 EFFECTS OF FP-d and FP-e POINT MUTATIONS ON pM-350 PROMOTER ACTIVITY IN RAW 264.7 CELLS.

13.4.1 Activity of pM-350 FP-d and FP-e point-mutant promoters in unstimulated RAW 264.7 cells.

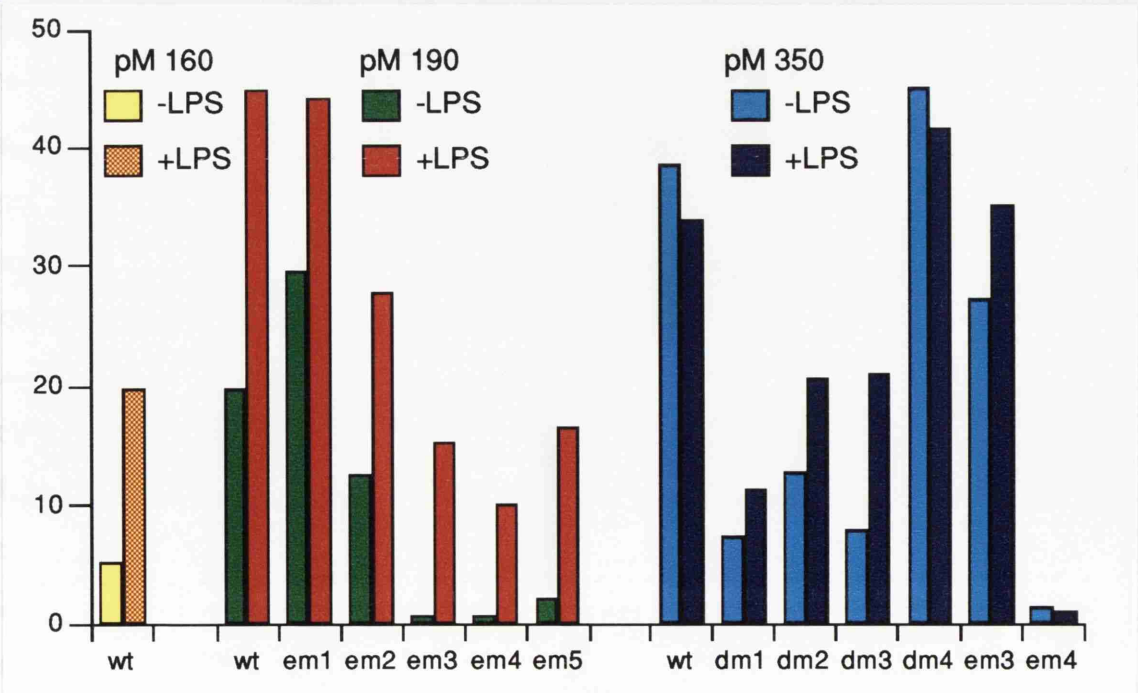
Figure 13.3: Effects of LPS on the expression of chimaeric mutant MIP1α promoter/ hGH gene constructs in RAW 264.7 cells.

Functional transient transfection studies of the activity of wild-type and mutant MIP1α promoter/hGH gene constructs in RAW 264.7 cells: effects of RAW CM (CM), RAW CM plus 2 μg/ml of LPS, or fresh 1% (v/v) FCS plus 2 μg/ml of LPS, on hGH gene expression. Constructs were co-transfected with reference plasmid pSV2APAP into RAW 264.7 cells and then incubated for 48 hours in medium containing fresh 10% (v/v) FCS; SPAP gene expression was assayed after 24 hours. Transfected cells were then PBS washed *in-situ*, and incubated for a further 20 hour test period in either RAW CM, RAW CM plus 2 μg/ml LPS, or 1% (v/v) FCS plus 2 μg/ml LPS, after which hGH gene expression was assayed. Assayed hGH gene expression was corrected for transfection efficiency (SPAP assay), and expressed relative to that of the pHSVTKGH positive control, which was given a value of 1 in RAW 264.7 cells in RAW CM. Values shown are the means of duplicate transfected plates in a single experiment. The data is presented in two forms:- **A**: For each construct, fold-induction of expression obtained in RAW CM plus LPS or 1% (v/v) FCS plus LPS is expressed in parentheses relative to the level of expression of the construct in RAW CM. Assayed promoter constructs are shown to the left of the table; **B**: Vertical axis, -fold expression of chimaeric wild-type and mutant constructs relative to the pHSVTKGH positive control. Horizontal axis: top of page, name of MIP1α promoter construct from which the tested mutants (bottom of page) are derived; wt, wild-type construct; em1-5 and dm1-4, mutants 1-5 and 1-4 inserted into the MIP1α promoter FP-e and FP-d footprinted sequences, respectively. Relative expression of each construct compared to pHSVTKGH in the absence- (-LPS) or presence- (+LPS) is as shown.

A

	RAWCM	RAWCM+LPS	1%FCS+LPS
pM-160	5.0	19.7 (3.9)	18.8 (3.8)
pM-190	19.7	44.7 (2.3)	44.2 (2.2)
pM-190em1	29.5	44.2 (1.5)	38.1 (1.3)
pM-190em2	12.6	27.9 (2.2)	27.1(2.2)
pM-190em3	0.6	15.2 (25.3)	12.3 (20.5)
pM-190em4	0.6	10.0 (16.7)	9.6 (16.0)
pM-190em5	2.0	16.6 (8.3)	25.2 (12.6)
pM-350	38.5	33.8 (0.9)	29.7 (0.8)
pM-350dm1	7.3	11.3 (1.5)	13.2 (1.8)
pM-350dm2	12.7	20.6 (1.6)	17.9 (1.4)
pM-350dm3	7.9	21.1 (2.7)	13.3 (1.7)
pM-350dm4	45.1	41.8 (0.9)	25.7 (0.6)
pM-350em3	27.3	35.1 (1.3)	24.2 (0.9)
pM-350em4	1.4	0.9 (0.6)	1.2 (0.9)

B



Although, in comparison with FP-a, pFP-c and dFP-c probes, FP-d probe binds C/EBP family members with low affinity *in-vitro* (in EMSAs, figure **11.2**), mutations dm1, dm2 and dm3, which each overlap the proposed C/EBP family binding site within FP-d (figure **13.1**), each reduce the basal activity of pM-350 in RAW 264.7 cells to 20-30% of wild-type activity (figure **13.3**), while mutation dm4, which lies outside the proposed FP-d C/EBP family binding site (figure **13.1**) does not significantly alter the basal activity of pM-350 (figure **13.3**). These results show that prevention of binding of C/EBP family members to the FP-d region significantly reduces the effects of the positive regulatory elements present in the distal region (-160 bp to -220 bp) of the MIP1α promoter on MIP1α promoter activity in unstimulated RAW 264.7 cells.

As described in section **13.3**, promoters pM-190em3 and pM-190em4 both have ~3% of wild-type promoter activity. However, when the same mutations are introduced into the pM-350 promoter, although pM-350em4 again has 3% of wild-type promoter activity in unstimulated RAW 264.7 cells, in contrast pM-350em3 has ~75% of wild-type activity in unstimulated RAW 264.7 cells (figure **13.3**). This is further confirmation of the presence of functionally important MIP1α promoter sequences upstream of -190 bp, and also suggests that mutations em3 and em4 do not simply affect the DNA binding of the same transcription factor(s); presumably, mutations em3 and em4 differentially affect communication between transcription factor(s) binding to the FP-f region and those binding to more proximal promoter sequences. Possible mechanisms for these observations will be addressed in the Discussion section.

13.4.2 Activity of pM-350 FP-d and FP-e point-mutant promoters in LPS stimulated RAW 264.7 cells.

As described earlier, the FP-e and FP-f regions of the MIP1α promoter contain strong constitutively-acting promoter elements, and the functional RAW 264.7 cell stable transfection data suggest that the effects of these elements mask the transient LPS-responsiveness of the MIP1α promoter conferred by elements proximal to -160 bp. Mutation dm4 does not overlap the proposed C/EBP family binding site in FP-d

(figure **11.1**), and only minimally affects the LPS-responsiveness of the pM-350 promoter over the 20-hour test period (figure **13.3**). However, mutations dm1, dm2 and dm3 do overlap the proposed FP-d C/EBP family binding site, and mutant promoters pM-350dm1, pM-350dm2 and pM-350dm3 are comparatively functionally responsive to LPS, pM-350dm3 being induced 2.7-fold over the test period (figure **13.3**). These results are consistent with the suggestion that prevention of binding of C/EBP family members to the FP-d region significantly reduces the effects of the positive regulatory elements present in the distal region (-160 bp to -220 bp) of the MIP1α promoter, thus unmasking LPS-responsiveness conferred by the proximal promoter. Further, these results demonstrate that binding of C/EBP family proteins to the FP-d region, while perhaps involved in-, is not essential for- LPS-responsiveness of the MIP1α promoter.

While the ***total activity*** of mutant promoter pM-350em3 is ~80% of that of wild-type pM-350 in LPS-stimulated RAW 264.7 cells, pM-350em4 has only ~4% of the total activity of pM-350 in LPS stimulated RAW 264.7 cells. In contrast, when the equivalent mutations were introduced into the pM-190 promoter, the total activities of pM-190em3 and pM-190em4 were similar in LPS-stimulated RAW 264.7 cells (figure **13.3** and section **13.3**). This is further evidence that mutations em3 and em4 do not simply affect the interaction of equivalent transcription factor(s) with the FP-e footprinted site of the MIP1α promoter. Possible mechanisms for these differences will be addressed in the Discussion section.

CHAPTER 14: MODULATION OF MIP1α GENE EXPRESSION IN RAW 264.7 CELLS BY INTERFERON-γ (IFN-γ), INTERFERON-α/β (IFNα/β) AND TRANSFORMING GROWTH FACTOR-β (TGFβ).

14.1 OVERVIEW.

14.1.1 Interferon gamma (IFN-γ).

IFN-γ is, like LPS, a potent macrophage activating factor (Chen *et al*, 1989), and may mediate at least some of its effects through the modulation of transcription of certain genes via κB-, IFN-γ activated sequence ("GAS")- and ISRE- *cis*-regulatory elements (Mirkovitch *et al*, 1992; Ohmori *et al*, 1993). Others have reported the modulation of MIP1α mRNA levels by IFN-γ in macrophages (Martin *et al*, 1991), and although the MIP1α promoter does not contain an identifiable "GAS" element (Mirkovitch *et al*, 1992) (figure **11.1c**), I have shown that NF-κB and other Rel-related proteins bind *in-vitro* to the MIP1α promoter at footprinted sites FP-b and FP-a (figures **11.10** and **11.11**), and site FP-a also contains 14/15 bp homology to the ISRE sequence (figure **13.1**), suggesting that MIP1α may be regulated by IFNγ at the level of transcription.

14.1.2 Interferons alpha and beta (IFNα/β).

Interferons α and β share many of the same functions, and are generally co-purified as IFNα/β (Chen *et al*, 1989; SIGMA chemical company). IFNα/β induces macrophage antiviral activity and, though less potent than IFNγ, has macrophage activating functions such as induction of cytotoxicity via release of reactive oxygen intermediates (Chen *et al*, 1989). IFNα/β modulates transcription of resposive genes, inclulding a subset of genes expressed in macrophages, via the interaction of the ISGF3 transcription factor with the ISRE *cis*-regulatory element (Fu, 1992; Schindler *et al*, 1992). ISGF3 (see Introduction) consists of ISGF3α and ISGF3γ, and many cell-types contain low levels of ISGF3γ, which can be induced by pretreatment of the cells with IFNγ and is then available for interaction with IFNα-induced ISGF3α (Fu, 1992; Schindler *et al*, 1992; Improta *et al*, 1992). As outlined above, the MIP1α promoter footprinted site FP-a contains a 14/15 bp homology with the ISRE sequence (figure **13.1**), which suggests that MIP1α may be regulated at the level of transcription by IFNα/β in responsive cell types.

14.1.3 Transforming growth factor beta (TGFβ).

The TGFβ proteins are a family of highly-related (structurally and functionally) cytokines, that share several properties with MIP1α, including inhibition of stem cell proliferation, and multiple roles in inflammation and wound healing (Keller *et al*,

1992; Rappolee *et al*, 1989). TGFb modulates the transcription of subsets of genes in responsive cells via DNA binding of the AP-1 transcription factor complex (Kim *et al*, 1989; Kim *et al*, 1990; Kerr *et al*, 1990).

AP-1 was originally identified as a complex binding to the TPA-responsive element (TRE), a motif able to confer phorbol ester-inducibililty on associated genes in mammalian cells (Gillespie, 1991). Although the MIP1α promoter contains no canonical TRE elements, I have shown that the MIP1α promoter FP-f probe binds an AP-1-like factor *in vitro* in EMSAs (figures **12.1-12.4**). Further, the 3' end of the FP-f probe sequence contains a sequence which is identical to an AP-1 binding site in the human TGFβ1 promoter, that is a TGFβ- (and phorbol ester-) responsive element (Kim *et al*, 1989; Kim *et al*, 1990) (figure **12.1b**). Hence, this suggests that the MIP1α promoter AP-1 binding site may be a TGFβ responsive element.

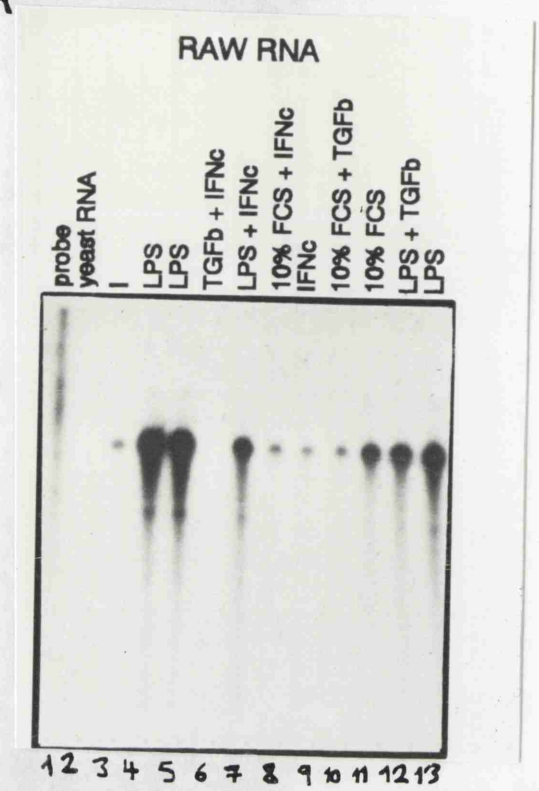
14.2 PRELIMINARY INVESTIGATION OF THE EFFECTS OF INTERFERON-γ (IFN-γ), INTERFERON-α/β (IFNα/β) AND TRANSFORMING GROWTH FACTOR-β (TGFβ) ON MIP1α mRNA LEVELS IN RAW 264.7 CELLS.

14.2.1 Negative modulation of MIP1α mRNA levels in RAW 264.7 cells by TGFβ and IFN-γ.

The effects of TGFβ and IFN-γ on MIP1α mRNA levels in RAW 264.7 cells were measured by the RNase protection assay using an antisense RNA riboprobe synthesized from the pSK-MIP1α plasmid (Table **6.1**). Prior to the cell stimulation, cells were incubated for 72 hours after replating in medium containing fresh 10% FCS. Cell stimulation was for 4 hours, during which the cell growth medium was RAW CM unless otherwise stated.

In unstimulated RAW 264.7 cells, MIP1α mRNA is at low basal levels (figure **14.1a**, lane 3), and its abundance is greatly increased by stimulation of the cells with either 2 μg/ml of LPS, or fresh cell growth medium containing fresh 10% FCS (figure **14.1a**, lanes 4, 5, 11, 13). While stimulation of the cells with IFN-γ only slightly reduces the basal levels of MIP1α mRNA (figure **14.1a**, lane 9), stimulation with

A


RAW RNA
probe
yeast RNA
–
LPS
LPS
TGFb + IFNc
LPS + IFNc
10% FCS + IFNc
IFNc
10% FCS + TGFb
10% FCS
LPS + TGFb
LPS
1 2 3 4 5 6 7 8 9 10 11 12 13

B

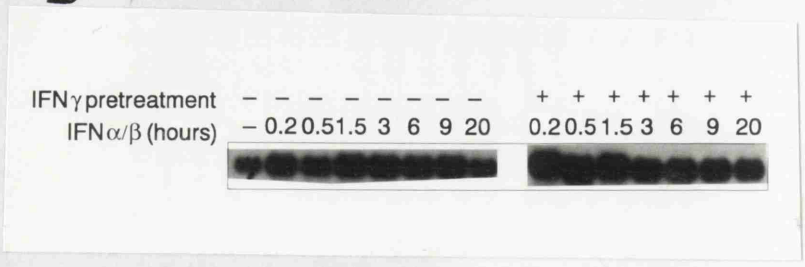

IFNγ pretreatment – – – – – – – – – + + + + + + +
IFNα/β (hours) – 0.2 0.5 1.5 3 6 9 20 0.2 0.5 1.5 3 6 9 20

Figure 14.1: Modulation of MIP1α RNA levels in RAW 264.7 cells by TGFβ1, IFNγ and IFNα/β, measured by RNase protection and Northern blotting analysis.

A: RNase protection analysis of total RNA prepared from RAW 264.7 cells stably transfected with chimaeric MIP1α promoter/hGH gene construct pM-900. Prior to the experiment, the RAW 264.7 cells were incubated for 3 days after replating in SLM containing fresh 10% (v/v) FCS. Total RNA was then prepared after the following treatment of the cells for 4 hours: Lane 3, no treatment control (RAW CM); lanes 4, 5 and 13, RAW CM plus 2 μg/ml of LPS; lane 6, RAW CM plus TGFβ1 (300 pM) and IFNγ (20 ng/ml); lane 7, RAW CM plus 2 μg/ml LPS and IFNγ (20 ng/ml); lane 8, fresh SLM plus fresh 10% (v/v) FCS and IFNγ (20 ng/ml); lane 9, RAW CM plus IFNγ (20 ng/ml); lane 10, fresh SLM plus fresh 10% FCS (v/v) and TGFβ1 (300 pM); lane 11, fresh SLM plus fresh 10% (v/v) FCS; lane 12, RAW CM plus 2 μg/ml LPS and TGFβ1 (300 pM). 20 μg of total RNA from each treatment and 20 mg of control yeast RNA were separately hybridized with the uniformly [α-^{32}P]-UTP-labelled 415 base antisense MIP1α RNA riboprobe (synthesized from linearized pSK-MIP1α plasmid template) at 45^{o}C in an RNase protection assay for 24 hours. Hybrids were then digested with an RNaseA/RNaseT1 mixture (5 μg/ml and 100 U/ml respectively) and protected RNA fragments resolved by denaturing PAGE and autoradiography. Lanes 1 and 2, yeast control RNA/ riboprobe mixture: mock-digested (lane 1) or RNase-digested (lane 2), as above. **B:** Northern blot of 20 μg of total cellular RNA prepared from RAW 264.7 cells after various experimental treatments. Filter were probed with [α-^{32}P]-UTP-labelled MIP1a antisense RNA riboprobe (see A). Prior to the experiment, the RAW 264.7 cells were incubated for 3 days after replating in SLM containing fresh 10% (v/v). Cells were incubated for a further 15 hours in RAW CM in the absence (-) or presence (+) of recombinant IFNγ (500 U/ml), prior to treatment for the indicated times (hrs) with IFNα/β (500 U/ml). Lane 1, untreated control RAW 264.7 cells in RAW CM.

IFN-γ plus TGFβ is sufficient to abolish MIP1α mRNA to below detectable levels (figure **14.1a**, lane 6). In the described experiments, I did not treat unstimulated RAW 264.7 cells with TGFβ alone; however, this experiment has been done in the laboratory (G.Graham, personal communication), and results in the abolition of detectable MIP1α mRNA in the treated RAW 264.7 cells. The abolition of detectable MIP1α mRNA in RAW 264.7 cells co-treated with IFN-γ plus TGFβ (figure **14.1a**, lane 6) is therefore probably mainly due to the TGFβ.

Co-treatment of RAW 264.7 cells with either TGFβ and fresh 10% FCS (figure **14.1a**, lane 10) or with IFN-γ and fresh 10 % (v/v) FCS (figure **14.1a**, lane 8) was sufficient to prevent the upregulation of MIP1α mRNA levels by 10% FCS. A similar, though less marked, response was observed upon co-treatment of RAW 264.7 cells with either of these factors and LPS (lanes 7 and 12), the lower response probably being due to the excessive amounts of LPS used (2 μg/ml). The significance of these results will be addressed in the Discussion.

14.2.2 MIP1α mRNA levels in RAW 264.7 cells may be upregulated in response to stimulation of the cells with IFNα/β.

The response of MIP1α mRNA levels to IFNα/β treatment of RAW 264.7 cells was measured by Northern blot analysis (figure **14.1b**), probed with the a riboprobe synthesized from the pSK-MIPcDNA plasmid (Table 1). Prior to the experiment, the RAW 264.7 cells were incubated for 72 hours after replating in cell growth medium containing fresh 10% (v/v) FCS. However, control (unstimulated) RAW 264.7 cell RNA samples were spoiled, and the control RAW 264.7 cell RNA sample used in this experiment was from another experiment, in which the cells had been pre-incubated for only 48 hours, not 72 hours. Hence, the control RAW 264.7 cell MIP1a mRNA levels (figure **14.1b**, lane 1) may be higher than would be expected.

After preincubation for 72 hours, RAW 264.7 cells were then pre-incubated for a further 15 hours in the presence (+) or absence (-) of IFN-γ, before stimulation over a timecourse with IFNα/β. The pre-incubation with IFN-γ was carried out because different cell types contain different levels of the ISGF3-γ component of the ISGF3 transcription factor, but levels of ISGF3-γ can be increαsed dramatically upon

pretreatment of cells with IFN-γ (Fu, 1992; Schindler *et al*, 1992; Improta *et al*, 1992).

Unfortunately, this experiment is not properly controlled, in that: a) the zero timepoints for each experiment were spoiled, so that the level of unstimulated MIP1α mRNA in this particular experiment is not known (see above), and the level of IFN-γ-stimulated MIP1α mRNA in the IFN-γ pre-treated cells prior to addition of IFN-α/β is not known; b) in a repeat of this experiment, control samples should be taken at each time-point, in order to account for the possible reduction in basal MIP1α mRNA levels in control cells over the 20 hour timecourse of the experiment. Nevertheless, given the results from the experiment shown in figure **14.1a**, wherein treatment of unstimulated RAW 264.7 cells with IFN-γ for 4 hours does not induce MIP1α mRNA above basal levels, the results from the experiment shown in figure **14.1b** suggest that IFNα/β does in fact rapidly induce MIP1α mRNA in RAW 264.7 cells. Further, as the levels of IFNα/β-induced MIP1α mRNA are greater in the IFN-γ-pretreated cells (figure **14.1b**), this suggests that 15 hr pre-treatment with IFN-γ upregulates the levels of ISGF3γ in RAW 264.7 cells.

14.3 FUNCTIONAL STUDIES: INVESTIGATION OF THE EFFECTS OF IFN-γ, IFNα/β AND TGFβ ON TRANSCRIPTION OF THE MIP1α GENE IN RAW 264.7 CELLS.

If IFN-γ treatment of RAW 264.7 cells affects MIP1α transcription, this effect is likely to be mediated by the ISRE in MIP1α promoter footprinted site FP-a, and the NF-κB/Rel family binding site in MIP1α promoter footprinted site FP-b (Ohmori *et al*, 1993). MIP1α mutant promoters containing the mutations am40, am41, am44, am48, bm30 and bm35 (figure **13.1** and Table **6.2**) would therefore be predicted to have a non-wild-type response to IFN-γ treatment of RAW 264.7 cells. Similarly, a transcriptional response of the MIP1α gene to IFNα/β would likely be mediated by the ISRE in FP-a, and mutations am40, am41, am44 and am48 would be expected to affect this response (figure **13.1** and Table **6.2**).

If the MIP1α gene responds transcriptionally to TGFβ treatment of RAW 264.7 cells, this is likely to be via the AP-1 transcription factor binding site overlapping the MIP1α promoter FP-e footprinted sequence. Mutation em1 (figure **13.1** and Table **6.2**) specifically mutates this putative AP-1 binding site, so would be expected to affect any response of the MIP1α promoter to TGFβ stimulation.

CHAPTER 15: NUCLEAR RUN-ON ANALYSIS : MEASURING THE RATES OF TRANSCRIPTIONAL INITIATION AND ELONGATION OF THE ENDOGENOUS MIP1α GENE AND CHIMAERIC MIP1α PROMOTER/ hGH GENE CONSTRUCTS IN RAW 264.7 CELLS.

15.1 NUCLEAR RUN-ONS VERSUS TRANSFECTION ASSAYS.

Functional cell transfection assays of chimaeric promoter/ reporter gene constructs allow an indirect measurement of the aggregate transcriptional activity of the reporter gene, conferred by the promoter deletion construct, over the time of the assay. For example, my own assays of MIP1α promoter deletion constructs were over a 20 hour test period, to allow for transcription and translation of the hGH reporter gene, and for secretion of the hGH protein from the cells. A disadvantage of transfection studies is that they do not necessarily reflect the effect of that element on transcription of the endogenous gene. Further, because of the long test period in transfection assays, which only measure an aggregate response, the kinetics and magnitude of the transcriptional response to cell stimulation remain unknown. In contrast, nuclear run-on analysis is a way of directly measuring the rate of transcription of endogenous-, and perhaps stably-transfected reporter-, genes at any particular instant in a given cell-type. Disadvantages of this assay are its technical difficulty, timescale and lack of sensitivity.

15.2 WHAT IS A NUCLEAR RUN-ON ?

A gene that is transcriptionally active in a particular cell-type is continually being traversed by RNA Polymerase II molecules, so that at any given moment, several different RNA Polymerase II molecules are at different points along its length. Thus, in a large population of such cells, each point along the actively-transcribed part of the gene is effectively being transcribed by the same number of RNA Polymerase II molecules at any given instant, which, upon isolation of cell nuclei under the right experimental conditions (chapter **7**) stop transcribing, due to lack of substrates, and are "frozen" to the gene at the point they have reached along the gene.

Nuclear run-on analysis involves incubation of isolated nuclei with the required RNA Polymerase II substrates, including [^{32}P-α]-UTP, so that "frozen" RNA Polymerase II molecules complete the round of transcription in which they are involved. Total RNA, containing ^{32}P-labelled elongated RNA, isolated from labelled nuclei is then incubated with DNA probes, immobilized on nitrocellulose filters, representing different sections of the gene whose transcription is being analyzed. In this way, nuclear run-ons can be used to examine the rate of transcriptional initiation or elongation of a gene at any given instant.

15.3 NUCLEAR RUN-ON ANALYSIS OF MIP1α GENE TRANSCRIPTION.

If nuclear run-ons were to show that rate of MIP1α gene transcriptional initiation, in response to LPS- or fresh FCS stimulation of RAW 264.7 cells, peaks at the same time as the maximal *in-vitro* DNA binding of the C/EBP family proteins forming complexes 1/a1 and 2/a2 with probes FP-a, pFP-c, dFP-c and FP-d in EMSAs (figure **11.9c**), this would strongly support the involvement of these complexes in MIP1α transcriptional induction.

In functional studies of MIP1α promoter deletion constructs in RAW 264.7 cells, although the pM-160 MIP1α promoter is responsive to LPS stimulation, pM-350, for example, is not LPS responsive over the test period (figures **10.3** and **13.3**). This could be because constitutively active promoter elements in the MIP1α promoter distal regulatory element (-160 bp to -220 bp) mask LPS-induced rapid

transcriptional up-regulation and repression mediated by MIP1α proximal promoter region (+36 bp to -160 bp), over the 20 hour test period.

Use of nuclear run-ons to measure the effects of LPS on the rate of transcriptional initiation of the endogenous MIP1α gene would indicate whether the LPS-mediated induction of transcription of the pM-160 MIP1α promoter construct is functionally relevant in the context of the endogenous gene. Further, if the technique is sensitive enough, RAW 264.7 cells stably transfected with constructs containing either the pM-900, pM-350 or pM-160 MIP1a promoter (Table **6.2**) could be used to compare the transcriptional response to LPS stimulation of these promoters with that of the endogenous MIP1α gene; though similarity of the response of a promoter construct and the endogenous MIP1α gene would not necessarily be informative, a markedly different response would show that essential promoter elements are absent from that construct. Nuclear run-on analysis of the transcriptional response of the endogenous MIP1a gene to IFNα/β, IFN-γ or TGFβ treatment of RAW 264.7 cells would also be informative.

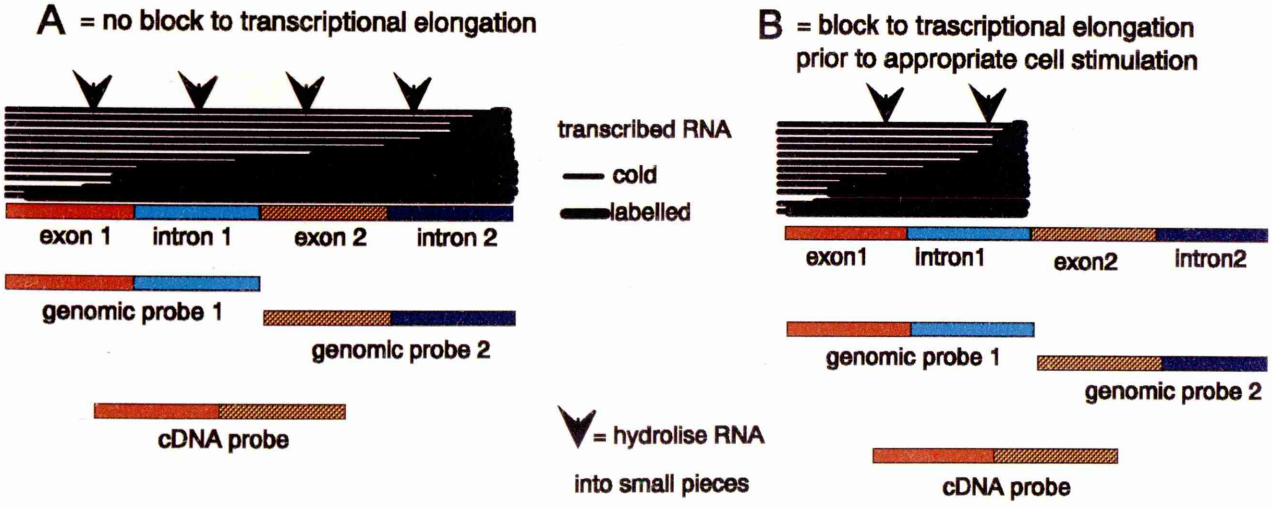
15.4 TRANSCRIPTIONAL INITIATION VERSUS TRANSCRIPTIONAL ELONGATION.

Modulation of the rate of transcription of early response genes in response to cell stimuli can occur at the level of both transcriptional initiation and/ or transcriptional elongation (Hayes *et al*, 1987; Collart *et al*, 1991). For example, premature termination of transcriptional elongation of the *c-fos* early-response gene in murine macrophages is relieved in a calcium-dependent manner (Collart *et al*, 1991). Nuclear run-on experiments investigating the rate of transcription of early-response genes such as MIP1α can distinguish between these two phenomena if appropriate DNA probes and partial hydrolysis of the labelled RNA samples are used in the nuclear run-on analysis (see below).

Figure **15.1** shows the 5' end of a hypothetical gene and the consequences of using different probes and partial hydrolysis of the labelled RNA samples, on the results obtained in nuclear run-ons. In figure **15.1a**, there is no block to

Figure 15.1: Model showing the advantages of using genomic probes rather than cDNA probes in nuclear run-on analysis of transcriptional initiation.

Exons 1 and 2 and introns 1 and 2 of the 5' end of a hypothetical gene are shown. Genomic probe 1 contains the DNA of exon 1 and intron 1, genomic probe 2 the DNA of exon 2 and intron 2, and the cDNA probe the DNA of exons 1 and 2, as shown. Transcribed RNA is denoted by horizontal lines above the cartoon of the gene. RNA Polymerase II molecules are travelling along the gene at the time of harvesting of the cells; the RNA transcribed by these individual molecules up to the time of harvesting of cells is denoted by narrow lines ("cold"), and the remaining RNA transcribed by these molecules in the nuclear run-on experiment is denoted by bold lines ("labelled"). Partial (random) hydrolysis of the resulting RNA prior to incubation of the RNA with the probe is denoted by vertical arrowheads above the horizontal RNA molecules. The gene is transcribed with no block to transcriptional elongation (**A**) or a block to transcriptional elongation at the end of intron 1 (**B**). Hypothetical experiments, in which cell stimulation leads to a 5-fold increase in transcriptional initiation (**A**) or a 5-fold increase in transcriptional elongation past the end of intron 1 (**B**) of this gene, are shown at the bottom of the page. Genomic probes 1 and 2 (probes 1 and 2) or the cDNA probe, are used in nuclear run-ons, as shown, and the expected -fold increase over unstimulated levels of RNA detected by each probe, in the absence (-) or presence (+) of hydrolysis of RNA prior to incubation with the probe,is as shown.



eg. Initiation increases 5-fold

RNA hydrolysis	-	+
	-fold increase of detected RNA	
probe 1	5	5
probe 2	5	5
cDNA probe	5	5

eg. Elongation increases 5-fold

RNA hydrolysis	-	+
	-fold increase of detected RNA	
probe 1	~5	1
probe 2	5	5
cDNA probe	~5	~5

transcriptional elongation, and when the rate of transcriptional initiation increases 5-fold, genomic probes 1 and 2 and the cDNA probe all detect a 5-fold increase in labelled RNA, whether or not the labelled run-on RNA is partially hydrolised prior to probing. However, in figure **15.1b**, cell-stimulation leads to a 5-fold increase in the rate of transcriptional elongation past a block at the end of intron 1, but no increase in the rate of transcriptional initiation. Without partial hydrolysis of run-off RNA, all three probes detect an approximately 5-fold increase in labelled RNA. However, partial hydrolysis of run-off RNA prior to probing allows the combined use of genomic probes 1 and 2 to distinguish between transcriptional initiation and transcriptional elongation, while the cDNA probe cannot distinguish between the two (figure **15.1b**).

15.5 NUCLEAR RUN-ON ANALYSIS OF MIP1α GENE TRANSCRIPTION IN RAW 264.7 CELLS.

15.5.1 Synthesis of probes.

In light of the above information, I constructed three probes, MNR1, MNR2 and MNR3 spanning the MIP1α gene (figure **15.2a**), to examine transcription of the endogenous MIP1α gene, and two probes, HGHNR1 and HGHNR2, that span the hGH gene (figure **15.2b**), to examine the levels of transcription of chimaeric MIP1α promoter/ hGH gene constructs in stably-transfected RAW 264.7 cells. All of these probes were subcloned into the pUC18 plasmid (Table **6.1**).

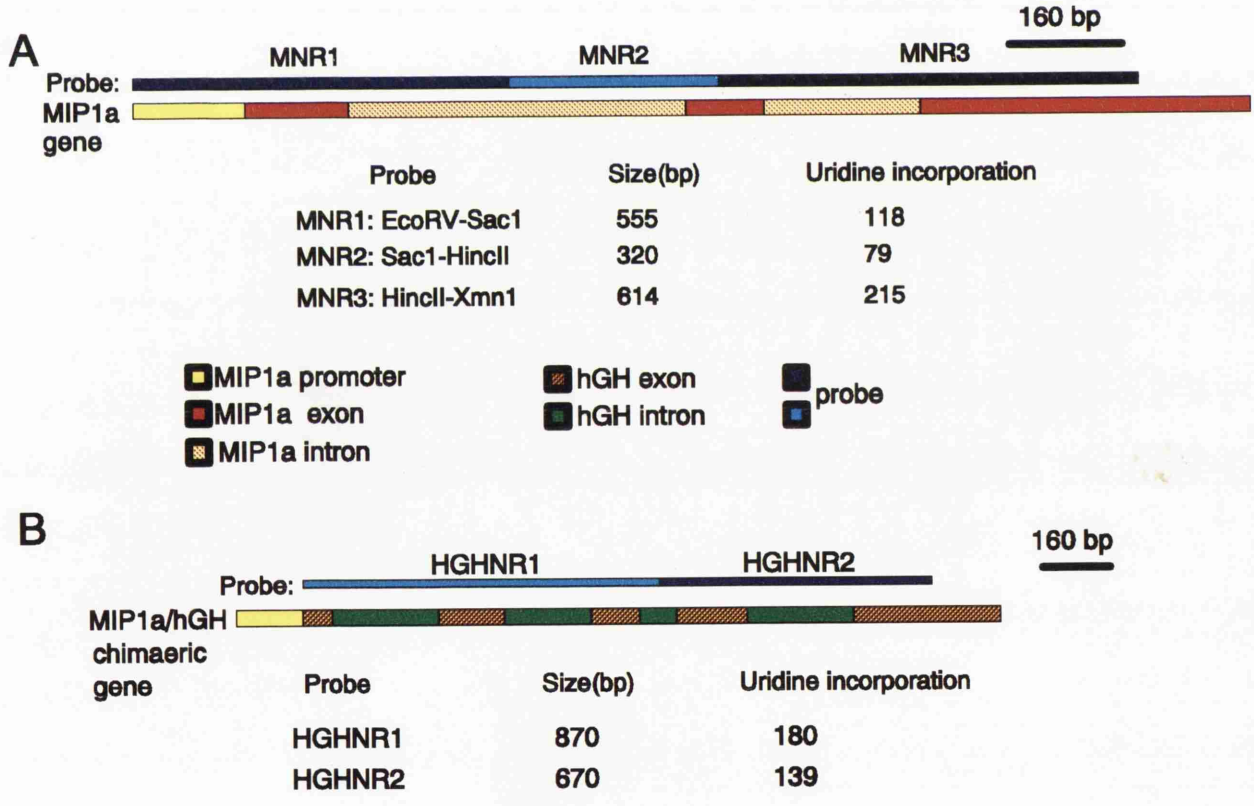
15.5.2 Nuclear run-on analysis of the endogenous MIP1α gene and stably transfected pM-900 MIP1α/ hGH reporter gene in RAW 264.7 cells.

In nuclear run-on experiments using nuclei from RAW 264.7 cells stably transfected with the pGHM-900 MIP1α promoter construct (Table **6.2**), a high level of labelling of TCA precipitable material (ie. RNA) was routinely obtained (>$2x10^6$ cpm/ 10 μCi input). The RNA was partially hydrolized before use, as described in chapter **7**, and discussed above.

MIP1α genomic probes MNR1-3- and hGH-specific genomic probe HGHNR2 (see figure **15.2**), along with negative control plasmid pUC18 and a positive control

Figure 15.2: Synthesis of genomic probes for nuclear run-on analysis of the rate of transcription of endogenous MIP1α- and chimaeric MIP1α promoter/hGH genes.

A: Schematic diagram of the MIP1α gene, showing MIP1α promoter-, exon- and intron sequences, and of contiguous DNA probes MNR1, MNR2 and MNR3 derived from the MIP1α gene-containing plasmid p18E7 by the indicated restriction digests, and cloned into pUC18. The coding-strand sequences of the MNR1-, 2- and 3- probes contain 118, 79 and 215 thymidine bases, respectively, and MIP1a RNA transcribed from these regions incorporates 118, 79 and 215 uridine bases respectively, as shown. **B**: Schematic diagram of the chimaeric MIP1α promoter/hGH gene, and of the contiguous DNA probes HGHNR1 and HGHNR2, derived from BamH1-Sma1 and Sma1-Sma1 digestion, respectively, of plasmid p0GH, and cloned into pUC18. The coding-strand sequences of HGHNR-1 and -2 probes contain 180- and 139 thymidine bases respectively, and hGH RNA transcribed from these regions of the chimaeric gene incorporates 180- and 139 uridine bases, respectively, as shown.

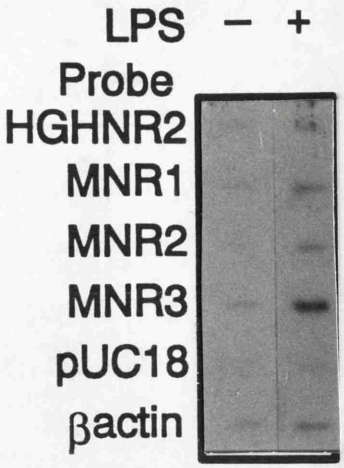


pUC18 plasmid containing the gene encoding β-actin, were immobilised on nitrocellulose and hybridized to labelled RNA from various nuclear extracts from the stably-transfected RAW 264.7 cells. As the β-actin gene is an early-response gene in monocytes (Mufson, 1990) and is rapidly transcriptionally induced in serum-stimulated fibroblasts (Stoflet *et al*, 1992), it is likely that LPS or fresh serum stimulation will rapidly transcriptionally induce the β-actin gene in RAW 264.7 cells. The experiment shown in figure **15.3** is a **preliminary** experiment, in which nuclei from unstimulated- were compared with those from LPS-stimulated pGHM-900-stably-transfected RAW 264.7 cells (as this was a preliminary experiment, I did not keep a note of the exact times of LPS stimulation). In this experiment, some hybridization of labelled RNA to the pUC18 probe is detected (figures **15.3a**), perhaps due to over-hydrolysis of RNA (ie. resulting in shorter RNA pieces with concomm/itt/antly less binding specificity). Nevertheless, the signal obtained with all the other probes is higher than that with pUC18, so that specific hybridization to these other probes has occurred. As expected, of the MIP1a probes, due to the greater number of uridine bases that can be incorporated into the MNR3 probe (figure **15.2a**), hybridisation to this probe is greatest (figure **15.3**). Densitometric quantitation shows that the rate of transcription of the β-actin gene is 4.5-fold higher in the LPS-stimulated- compared to the unstimulated RAW 264.7 cells (figure **15.3c**) (elongation and initiation were not distinguished using the β-actin probe). Similarly, the rate of transcription measured by MIP1a gene probes MNR1 and MNR2 was increased between 4 to 6-fold by LPS stimulation of RAW 264.7 cells (figure **15.3c**), suggesting that LPS stimulation increases the rate of transcriptional initiation of the endogenous MIP1α gene in RAW 264.7 cells. However, the rate of endogenous MIP1a gene transcription measured by the MNR3 probe is increased ~12-fold by LPS stimulation (figure **15.3c**), suggesting that a block, somewhere in the ~600 bp region spanned by the MNR3 probe (figure **15.2a**), to transcriptional elongation of the endogenous MIP1a gene, is present in unstimulated RAW 264.7 cells and is relieved in an LPS-dependent manner.

Figure 15.3: The effects of LPS treatment of RAW 264.7 cells on transcriptional initiation and elongation of the MIP1α gene.

Nuclear run-on analysis of the endogenous MIP1α gene and of the chimaeric pM-900 MIP1α promoter/ hGH gene, using nuclei isolated from RAW 264.7 cells stably-transfected with the pM-900/ hGH gene construct. **A**: Stable transfectants were grown in medium containing fresh 10% (v/v) FCS for 72 hours, then incubated in RAW CM in the absence (-) or presence (+) of 2 μg/ml of LPS for an unremembered period of between 2-8 hours before isolation of nuclei. The indicated plasmid DNA probes, where HGHNR2 is an hGH gene genomic probe, MNR1-3 are MIP1α gene genomic probes, pUC18 plasmid is a negative control, and β-actin is a murine β-actin gene cDNA probe positive control, were immobilized on nitrocellulose filters then inubated for 48 hours at 65^{o}C with partially-hydrolized RNA from nuclear run-on reactions. Washed filters were visualized by overnight autoradiography. **B**: The autoradiograph shown in (**A**) was analysed by densitometry, and relative abundance (ODxMM^{2}) of the signal obtained with each probe in lane 1 (-LPS) and lane 2 (+LPS) is shown. **C**: The relative abundance (ODxmm^{2}) and corrected relative abundance (corrected ODxmm^{2}: obtained by subtracting the value obtained with the negative control pUC18 plasmid probe for that lane), of the signal obtained with each probe in lane 1 (-LPS) and lane 2 (+LPS) is shown. The -fold induction in abundance of the corrected signal obtained with each probe in the presence- compared to the absence of LPS stimulation is shown.

A



B

Lane 1 -LPS

	Band #	Rf	Standard	Peak OD	Quantity ODxMM	Quantity ODxMM^2	Rel Quantity Percent
HGHNR2	1 - 1	0.039		0.03	0.059	0.261	7.7
MNR1	1 - 2	0.221		0.04	0.080	0.360	10.5
MNR2	1 - 3	0.406		0.03	0.064	0.241	8.5
MNR3	1 - 4	0.584		0.06	0.108	0.491	14.2
pUC18	1 - 5	0.797		0.03	0.054	0.198	7.2
βActin	1 - 6	0.947		0.04	0.058	0.258	7.7

Lane 2 +LPS

	Band #	Rf	Standard	Peak OD	Quantity ODxMM	Quantity ODxMM^2	Rel Quantity Percent
HGHNR2	2 - 1	0.052		0.18	0.331	1.403	11.2
MNR1	2 - 2	0.237		0.13	0.226	1.305	7.7
MNR2	2 - 3	0.415		0.12	0.205	0.926	7.0
MNR3	2 - 4	0.592		0.66	0.868	4.138	29.4
pUC18	2 - 5	0.777		0.06	0.133	0.678	4.5
βActin	2 - 6	0.951		0.12	0.179	0.950	6.1

C

	-LPS		+LPS		-fold LPS induction
	ODx mm^2	corrected ODx mm^2	ODx mm^2	corrected ODx mm^2	
HGHNR2	O.261	0.063	1.403	0.678	10.8
MNR1	0.360	0.162	1.305	0.672	4.1
MNR2	0.241	0.043	0.926	0.248	5.8
MNR3	0.491	0.293	4.138	3.46	11.8
pUC18	0.198	0.0	0.678	0.0	0.0
βActin	0.258	0.06	0.950	0.272	4.5

Interestingly, the weak *in-vivo* DNase1 hypersensitive site that maps approximately to intron 2 of the MIP1α gene in unstimulated J774.2 cells (figure **9.3b**), is within the region of DNA contained in the MNR3 probe (figures **8.7** and **15.2a**, and Table **6.1**), suggesting a possible functional connection between the block to transcriptional elongation and the weak DNase1 hypersensitive region in intron 2 of the MIP1α gene, in unstimulated macrophage cell-lines.

The rate of transcription of the hGH gene mediated by the pM-900 MIP1α promoter, as measured by probe HGHNR2 (figure **15.2b**), was increased ~11-fold in the LPS-stimulated- compared to the unstimulated RAW 264.7 cells (figure **15.3c**). As the measured rate of transcriptional initiation of the endogenous MIP1 α gene, as measured by the MNR1 probe (figure **15.2a**), was only increased ~4-6-fold by LPS stimulation of RAW 264.7 cells (figure **15.3c**), this suggests that the pM-900 promoter lacks negative transcriptional-regulatory elements that regulate the endogenous MIP1a gene in RAW 264.7 cells.

PART 4: DISCUSSION.

CHAPTER 16: DISCUSSION OF RESULTS.

16.1 CELL GROWTH CONDITIONS.

The MIP1α gene is an early-response gene in RAW 264.7 cells, as MIP1α RNA is rapidly and transiently induced in these cells by both fresh 10% (v/v) FCS and LPS. A difference between the fresh serum- and LPS inductions was that MIP1α mRNA was back to basal levels 24 hours after LPS induction, but after fresh serum induction, the cells had to be incubated for ~72 hours until mRNA levels were back to basal levels. Hence, "uninduced" RAW 264.7 cells were those that had been incubated for 72 hours after replating in cell growth medium containing fresh 10% (v/v) FCS, and the medium from these cells was termed RAW CM. Endogenous MIP1α mRNA levels were at low basal levels in these cells, and RAW CM was used in RAW 264.7 cell transfection studies to measure the basal level of MIP1α promoter construct activity in these cells. Presented experiments show that it is specifically *fresh* FCS that induces MIP1α mRNA in RAW 264.7 cells. This suggests that the FCS contains certain growth factor(s) that are either in limiting concentrations, or that are particularly labile.

I decided to use RAW CM to define the basal levels of MIP1α gene expression in RAW 264.7 cells, because the conditions for growth of the cells are then constant throughout the experiment. In contrast, in serum-free medium, another possible option to define MIP1α basal expression, the morphology of the RAW 264.7 cells changes, and MIP1α mRNA levels are rapidly induced (M.Grove and M.Plumb, unpublished results).

Due to the limited number of signal transduction pathways employed by cells, up-regulation of early-response gene mRNA production may sometimes occur as an unavoidable consequence of the activation of a particular signal transduction pathway, without concomittant protein production (Gillespie, 1991). For example, in monocytes, IL-1β mRNA can be transiently induced by adherence of the cells to plastic, without induction of IL-1β protein synthesis, while LPS stimulation results in a similar transient mRNA induction, but the mRNA is efficiently translated

(Schindler *et al*, 1990). It is therefore possible that MIP1α mRNA induced in serum-free conditions is not translated.

16.2 MIP1α GENE TRANSCRIPTIONAL REGULATORY SEQUENCES.

16.2.1 DNaseI hypersensitivity studies of the MIP1α gene locus in J774.2 and RAW 264.7 cells.

In unstimulated J774.2-, WEIHI- and RAW 264.7 cells, the proximal MIP1α promoter (~+1 to -350 bps) is DNaseI hypersensitive, correlating with an active chromosomal conformation and the presence of transcription factors binding to those DNA sequences *in-vivo*. Hence, like the promoters of other early-response genes in expressing cell types (Herrera *et al*, 1989; Gille *et al*, 1992), the MIP1α promoter is "poised" prior to cell stimulation.

A second, very weak region of DNaseI hypersensitivity, which maps to the second intron of the MIP1α gene, was observed in unstimulated J774.2 cell nuclei (RAW 264.7 cells were not tested). It is known that the translational position of nucleosome core particles on DNA is dependent on the sequence of that DNA (Drew *et al*, 1987), largely due to the sequence-specificity of DNA bending, so that sequences such as AAA or TTT prefer to place their minor groove edges along the inside of a curve, while GGG, CCC or GGC prefer to place their minor-groove edges along the outside (Drew *et al*, 1987). Further, certain repetitive sequences such as poly[(dA).(dT)] or poly[(dG).(dC)] will not fold around nucleosomal core particles (Drew *et al*, 1987). As the MIP1α gene second intron contains several stretches of repetitive sequences (figure **8.7**), it is possible that part of this region is not efficiently incorporated into nucleosomes, so that the DNA is more accessible than bulk chromatin, resulting in weak DNaseI hypersensitivity at that site. Although the functional significance of this site has not yet been tested, in my preliminary nuclear run-on experiments using probe MNR3, which encompasses the MIP1α second intron sequences, a block to MIP1α transcriptional elongation was detected in unstimulated RAW 264.7 cells. Hence, the region of weak DNaseI hypersensitivity is

within the region of chromatin where the block to transcriptional elongation occurs, suggesting a possible functional correlation between these two observations.

Stimulation of RAW 264.7 cells for 12 hours with LPS did not appear to induce any additional DNase1 hypersensitive sites within a 5 kbp MIP1α promoter region. However, as MIP1α mRNA is rapidly induced within 30' to 1 hour by LPS or fresh FCS in RAW 264.7 cells, transient induction of transcription factor binding (and concomitant induction of DNase1 hypersensitivity) to MIP1α gene regulatory sequences could occur during this period, which was undetectable 12 hours after LPS stimulation (Verdin *et al*, 1993; Cockerill *et al*, 1993), at which time MIP1α mRNA levels are declining.

As discussed in chapter **5**, DNA replication is generally required in order for transcription factors to displace non-acetylated- but not acetylated nucleosomes from DNA (Lee *et al*, 1993), and in so doing create a DNase1 hypersensitive site. Further, in active genes, nucleosomes over the promoter appear to be acetylated, while those outwith this region are not (Turner *et al*, 1990). This suggests that LPS stimulation might rapidly (within ~30 minutes) induce additional MIP1α proximal promoter DNase1 hypersensitivity, but that induction of DNase1 hypersensitivity of more distant regions of chromatin would be less rapid (eg. after ~4-6 hours, when induced MIP1α mRNA levels peak) (c.f. Cockerill *et al*, 1993; Verdin *et al*, 1993). However, the MIP1α promoter functional transfection data suggests that within a 936 bp region (+36 bp to -900), only the ~300 bp region that is DNase1 hypersensitive in unstimulated RAW 264.7 and J774.2 cells is functionally important, suggesting the induction of further DNase1 hypersensitivity of the MIP1α promoter in these cells is not required for the transcriptional response to LPS.

16.2.2 Identification of MIP1α gene regulatory sequences outside the ~10 kbp region studied.

If the ~300 bp MIP1α proximal promoter region is the only *cis*-acting region of DNA that affects the expression of the MIP1α gene in macrophages, it might be expected that this region of DNA would function as a chromatin boundary domain and/or an LCR (see chapter **5**). This could be tested in functional studies of MIP1α

promoter/ hGH gene chimaeric constructs stably transfected into RAW 264.7 cells, as if stably-integrated constructs in single-cell clones were expressed in a position-of-integration-independent and copy-number-dependent manner, this would indicate that the MIP1α promoter region contains boundary region/ LCR function; if not, this would suggest that other *cis*-acting sequences are required for the correct expression of the MIP1α gene in macrophages.

As the expression of several members of the RANTES/SIS gene family, including MIP1α and MIP1β, appear to be restricted to cells of hemopoietic origin (my data, plus see chapter **1**), that the RANTES/SIS gene family is tightly clustered on murine chromosome 11q11-11q21 or human chromosome 17q11-17q21 (Wilson *et al*, 1990; Irving *et al*, 1990; Miller *et al*, 1990; Donlon *et al*, 1989), that two of the human MIP1α and MIP1β genes are only 14 kbp apart (Irving *et al*, 1990), and that the MIP1α and MIP1β genes are co-regulated by most cell-stimuli (Schall, 1991), this suggests that the MIP1α gene is likely to share distant regulatory elements with the MIP1β gene and possibly other RANTES/SIS family genes. If this were the case, by analogy with the β-globin gene locus (Forrester *et al*, 1990), it might be expected that the MIP1α gene is situated in a large region of DNase1-sensitive (not hypersensitive-) chromatin in macrophages, a theory that could be experimentally tested.

16.2.3 The significance of the lack of MIP1α promoter DNase1 hypersensitivity in non-haemopoietic cell types.

The endonuclease accessibility of the MIP1α promoter is cell-specific, correlating with the presence or absence of detectable MIP1α mRNA, and as the promoters of early-response genes are constitutively bound by transcription factors in expressing cell types (Herrera *et al*, 1989; Gille *et al*, 1992), this suggests that the MIP1α gene is not an early-response gene in non-expressing cell types. However, this does not preclude that the chromatin of the MIP1α locus could be reprogrammed, concommitant with DNA replication, by treating non-expressing cells with the appropriate stimulus.

It is not yet known whether the MIP1α gene locus is DNase1 sensitive, which would suggest that the gene is potentially active, in non-expressing cell types. If it

were, the gene would be replicated early in S-phase in these cells (Forrester *et al*, 1990), allowing for rapid reprogramming of the chromatin status of the MIP1α gene promoter upon the appropriate cell stimulation. In support of this, there is an isolated report of induction of the human MIP1a gene LD78α in primary fibroblasts, by 5 hrs stimulation with PMA (Nakao *et al*, 1990). A similar experiment was conducted in this laboratory using Swiss 3T3 fibroblasts, but with negative results (M.Plumb, personal communication). However, there is great heterogeneity in different fibroblast cell isolates; for example murine 3T3 (Swiss and L1) cells contain very low/ undetectable levels of any of the C/EBP family of transcription factors (Cao *et al*, 1991 and my results), while L cells and STO cells contain readily detectable levels of several C/EBP family proteins (Roman *et al*, 1990 and my results). As I have shown that C/EBP family members appear to play a role in the regulation of transcription of the MIP1α gene, this suggests that different isolates of fibroblasts could have different potential to express the MIP1α gene.

16.3 REGULATION OF MIP1α GENE TRANSCRIPTION BY C/EBP FAMILY PROTEINS.

16.3.1 Are any proteins other than C/EBP family proteins present in C/EBP-related complexes 1/a1, 2/a2 and 3/a3 ?

The leucine zipper (LZ) of bZIP proteins contains conserved residues other than those required for dimerization, suggesting that the LZ may allow dimerization of bZIP proteins with heterologous proteins (Johnson et al, 1989). In keeping with this, the NF-κB p50 and p65 proteins can physically interact with C/EBPβ, and the interaction involves the bZIP region of C/EBPβ and the RH domain of p50 and p65 (Stein *et al*, 1993; LeClair *et al*, 1992). Further, c-Rel, p50 and p65 enhance transcription mediated by C/EBPα, C/EBPβ or C/EBPδ from a C/EBP family DNA recognition site (Stein *et al*, 1993). This correlates with EMSA studies, in which NF-κB p65 greatly stimulates C/EBPα, C/EBPβ and C/EBPδ binding to a C/EBP probe, although there is no corresponding change in mobility of the C/EBP family proteins

in these EMSAs, so that the C/EBP family/ p65 protein-protein interactions can only be inferrred from these EMSAs (Stein *et al*, 1993).

Because C/EBP family protein binding to the MIP1α promoter sites FP-a, pFP-c, dFP-c and FP-d is not reduced by κBpd or FP-b competitors, this suggests that there is no significant stimulation of C/EBP family proteins binding to these probes by Rel family proteins present in RAW 264.7 cell nuclear extracts. One explanation for this may be that C/EBP family proteins are more abundant than Rel family proteins in RAW 264.7 cells. However, as the MIP1α gene proximal promoter contains at least one Rel family protein binding site (FP-b) flanked by several C/EBP family protein binding sites, interactions between these two transcription factor families is probably important in the *in-vivo* regulation of MIP1α gene transcription in RAW 264.7 cells.

In addition to interaction with Rel family proteins, the specific DNA binding activity of C/EBP family proteins *in-vitro* is significantly enhanced by the addition of small basic polypeptides or the basic DNA binding domain of the c-Fos protein (Bannister *et al*, 1992). This raises the possibility that the 1/a1, 2/a2 and 3/a3 C/EBP-related complexes detected in EMSAs with probes FP-a, pFP-c, dFP-c and FP-d, contain non-DNA-binding protein(s), interacting via a basic region with the DNA binding C/EBP family proteins.

In addition to complexes 1/a1, 2/a2 and 3/a3, in EMSAs using nuclear extracts from unstimulated RAW 264.7 cells, three faster-migrating complexes are formed with pFP-c probe, and these complexes, similarly to complex 3/a3, are significantly decreased in abundance in nuclear extracts from 5 hr LPS-stimulated RAW 264.7 cells. Only two- and none-, respectively, of these faster migrating complexes are formed with nuclear extracts from MEL- and STO cells. These complexes are strongly competed by FP-a competitor (figures **11.4b** and **11.8a**), but are not formed using FP-a probe; however, they are probably C/EBP-related, as the pFP-c probe does not contain any other observable homology to the FP-a site. It is possible, though perhaps unlikely, that while complexes 1/a1, 2/a2 and 3/a3 contain C/EBP proteins complexed with other non-DNA binding protein(s), which increases their affinity for the probes used, probe pFP-c is able to bind homo- and heterodimers of C/EBP

proteins uncomplexed with these other protein(s). As these complexes have relatively low affinity for the pFP-c probe, compared to complex 1/a1 formed with this probe (see below), their significance is unclear.

16.3.2 Which C/EBP family protein(s) are present in complexes 1/a1, 2/a2 and 3/a3 ?

There are at least four C/EBP family proteins, each of which can homodimerise and heterodimerize with other family members (Akira *et al*, 1990; Poll *et al*, 1990; Roman *et al*, 1990; Williams *et al*, 1991). Complexes 1/a1, 2/a2 and 3/a3 can form with nuclear extracts from RAW 264.7-, STO- and MEL cells, suggesting that these complexes contain C/EBP family proteins that are expressed in multiple cell types, such as C/EBPβ, C/EBPγ and C/EBPδ (Akira *et al*, 1990; Cao *et al*, 1991; Chang *et al*, 1990; Descombes *et al*, 1990; Poll *et al*, 1990; Roman *et al*, 1990; Kinoshita *et al*, 1992; Williams *et al*, 1991), and as C/EBPβ and C/EBPδ are expressed in macrophage cell lines, are induced by LPS, and are of similar molecular weight to one protein component detected in complexes 1/a1 and 2/a2 (Akira *et al*, 1990; Chang *et al*, 1990; Kinoshita *et al*, 1992; Scott *et al*, 1992), this suggests that one or both of these proteins is present in complexes 1/a1 and/or 2/a2.

The C/EBPβ gene is conserved between species, and encodes a single mRNA which contains three potential translational start AUGs (Descombes *et al*, 1990; Williams *et al*, 1991); the first of these is contained in a poor, the second- in a moderate- and the third in a perfect Kozak sequence, resulting in leaky ribosome scanning, and the synthesis of three different C/EBPβ proteins (Cao *et al*, 1991; Descombes *et al*, 1990; Hsu *et al*, 1993), which I shall henceforth refer to as C/EBPβ1, C/EBPβ2 and C/EBPβ3, respectively, in descending order of molecular size. The murine C/EBPδ mRNA also contains three potential translational start AUG codons (Cao *et al*, 1991), but the most 5' of these is in an almost perfect Kozak sequence, while the second is in a moderate- and the third in a poor Kozak sequence, suggesting that only the most 5' of the AUG sequences is utilized as a translational start site (Cao *et al*, 1991; Descombes *et al*, 1990).

Unlike the much smaller C/EBPβ3 protein, the molecular sizes of the C/EBPβ1, C/EBPβ2 and C/EBPδ proteins are very similar (Cao *et al*, 1991; Descombes *et al*, 1990; Hsu *et al*, 1993). Although electrophoretic mobility in EMSAs does not always reflect molecular size, in EMSAs using C/EBP probe with recombinant C/EBPβ2 and C/EBPβ3 proteins, slow-, intermediate- and faster-migrating complexes, containing C/EBPβ2 homodimers, C/EBPβ2-C/EBPβ3 heterodimers and C/EBPβ3 homodimers, respectively, are formed. This suggests that homo- and heterodimers of C/EBPβ1 and/or C/EBPβ2 and/or C/EBPδ are likely to migrate at a similar rate in EMSAs. As three differentially-migrating C/EBP-related complexes 1/a1, 2/a2 and 3/a3 are formed with MIP1α proximal promoter C/EBP family binding probes, this suggests that complex 1/a1 may contain C/EBPβ1 and/or C/EBPβ2 and/or C/EBPδ, while complex 2/a2 contains heterodimers between C/EBPβ3 and C/EBPβ1 and/or C/EBPβ2 and/or C/EBPδ, and complex 3/a3 contains homodimers of C/EBPβ3. Although in UV-crosslinking studies the only C/EBP family protein(s) present in complexes 1/a1 and 2/a2 observed to bind to the FP-a probe was of a similar size to C/EBPβ1, C/EBPβ2 or C/EBPδ, a possible reason for this may be that in heteromeric complexes, C/EBPβ3 binds to the non-coding strand of FP-a, in which only one BrdU nucleotide is present in the FP-a C/EBP family binding site. Unfortunately, I did not try UV-crosslinking of complex 3/a3 to probe FP-a, which would address this issue.

C/EBPβ1 and C/EBPβ2 proteins are both positive transcriptional regulators, though because they are differentially syntheisized in different cell types, this suggests that there is some unknown difference between them (Hsu *et al*, 1993; Descombes *et al*, 1990). C/EBPδ protein is also a positive transcriptional regulator, and these proteins can homodimerize and heterodimerize and transcriptionally synergize (Kinoshita *et al*, 1992; Ramji *et al*, 1992). In contrast, C/EBPβ3 lacks the N-terminal transcriptional activation domain posessed by these other proteins, and is a repressor of transcription (Descombes *et al*, 1990). This would suggest that, of the C/EBP-related complexes formed with MIP1α promoter C/EBP-family binding sites, complex 1/a1 is a strong positive regulator, complex 2/a2 is a weak positive regulator and complex 3/a3 is a negative regulator of MIP1α gene transcription. This is in

keeping with the EMSA data presented in chapter **11**, which implicated complex 1/a1, and to a lesser extent a2, in positive LPS-responsiveness-, and complex 3/a3 in negative regulation of the MIP1α promoter in RAW 264.7 cells.

Because macrophages contain C/EBPδ and C/EBPβ1 and/or C/EBPβ2, and that these three proteins can homo- and heterodimerize without affecting DNA binding, and that they are of similar molecular size, so probably can't be distinguished in EMSAs, where I have referred to C/EBPβ1 in the following discussion, this may equally apply to C/EBPβ1 and/or C/EBPβ2 and/or C/EBPδ, unless otherwise stated.

16.3.3 Model for the regulation of transcription of the MIP1α gene in RAW 264.7 cells by C/EBP family proteins.

It is apparent from sequence comparisons that the MIP1α proximal promoter contains two types of C/EBP family binding site, as FP-a and dFP-c are homologous to C/EBP-, mGPE-1 and Albumin promoter site D sites (Landschultz *et al*, 1988; Nishizawa *et al*, 1990; Descombes *et al*, 1990), whereas pFP-c is strongly- and FP-d weakly- homologous to the AGP/EBP-A C/EBP family binding site (Lee *et al*, 1993) (figure **16.1**). This difference is consistent with the pattern of C/EBP family complexes formed with these different probes in EMSAs: nuclear extracts from unstimulated RAW 264.7 cells and FP-a, dPF-c, C/EBP or mGPE-1 probes form equivalent levels of complexes 2/a2 and 3/a3, but very low levels of complex 1/a1 (figure **11.4a**); in contrast, although much greater levels of 1/a1, 2/a2 and 3/a3 complexes are formed with pFP-c probe compared to FP-d probe with unstimulated RAW 264.7 cell nuclear extracts, both share a binding pattern distinct from FP-a and dFP-c, in that the relative levels of complexes 1/a1, 2/a2 and 3/a3 are equivalent with probes pFP-c and FP-d (figure **11.4a**).

An advantage of EMSAs is that, within a particular nuclear extract, it is theoretically possible to detect all of the possible transcription factor complexes that can bind to a particular DNA site. Obviously, however, *in-vivo* only one of these complexes binds to that site at any one time. I propose that *in-vivo* in unstimulated RAW 264.7 cells, complex 1/a1 (C/EBPβ1) forms at sites pFP-c and FP-d, while complexes 2/a2 (C/EBPβ1/β3) or 3/a3 (C/EBPβ3) form at sites FP-a and dFP-c,

Figure 16.1: Sequence alignment of MIP1α promoter C/EBP family binding sites with documented C/EBP family binding sites

A: Sequence comparisons of MIP1α promoter sites FP-a and dFP-c with Albumin promoter site D (Mueller **et al**, 1990), murine G-CSF gene promoter site mGPE-1 (Nishizawa *et al*, 1990), human G-CSF gene promoter site hG-CSF (Shannon *et al*, 1990) and C/EBP (Landschultz *et al*, 1988). **B**: Sequence comparisons of MIP1a promoter sites pFP-c and FP-d with the alpha-acid glycoprotein gene promoter site AGP/EBP-A (Lee *et al*, 1993).

A

Albumin site D	**ATTTTGTAAT**
dFP-c	**gTTgTGTAAT**
FP-a	**gaTgaGgAAa**
mGPE-1 (nc)	**tTTgTGaAAT**
hG-CSF (nc)	**ATTgTGgAAT**
C/EBP	**ATTgcGcAAT**

B

AGP/EBP-A (c)	**ATCGTGTCATAA**
pFP-c (nc)	**ATgGTGTCATgA**
FP-d (c)	**ccCcTGTggTcA**

resulting in strong positive regulation of transcription mediated by sites pFP-c and FP-d and either weak positive- or negative transcriptional regulation mediated by sites FP-a and dFP-c. If more than one transcription factor complex is available to bind a particular promoter site *in-vivo*, several factors influence which of these complexes binds to the promoter (see chapter **3**), one of which is the relative **affinity** (see below) of each complex for the promoter site. Hence, I am proposing that of C/EBP-related complexes 1/a1, 2/a2 and 3/a3, complex 1/a1 has higher affinity than 2/a2 or 3/a3 for sites pFP-c and FP-d, while complexes 2/a2 and 3/a3 have higher affinity than complex 1/a1 for sites FP-a and dFP-c. Below, I will present evidence to support this model.

16.3.4 Specificity, affinity and abundance.

In order to discuss *affinity*, it is important to distinguish it from *specificity* and *abundance*, as follows: Imagine two hypothetical transcription factors T1 and T2 which bind a probe P1 containing the DNA core sequence CGCGCG; in EMSAs using probe P1, if both complexes T1 and T2 are competed by 25-fold excess of competitor P1 but not by 500-fold excess of a competitor lacking the CGCGCG site, then binding of T1 and T2 to probe P1 is ***specific***. In EMSAs, because of the large excess of probe used, if the EMSA reaction is incubated for sufficient time, binding of transcription factors T1 and T2 to the P1 probe will reach equilibrium, and essentially every available copy of T1 and T2 will be bound to the probe; thus, if the nuclear extract used contains equivalent amounts of T1 and T2, complexes T1 and T2 will form in equal ***abundance***. However, if over the time-period of the assay, a 5-fold excess of P1 competitor competes for binding of T1, but a 25-fold excess of P1 is required to compete for binding of T2, this means that T2 binds with 5-fold greater ***affinity*** than T1 to the P1 probe; this is due to greater stability of T2-P1- compared to T1-P1 complexes. Hence, the abundance of a particular complex formed in EMSAs does not necessarily reflect the affinity of that complex for the probe. Furthermore, in the absence of other influences (see chapter **3**), if equivalent amounts of complex T1 and T2 were present in the nucleus of a particular cell, a promoter containing site P1 would be bound by complex T2 rather than complex T1.

16.3.5 Affinity of different C/EBP-related complexes for MIP1α promoter sites FP-a, pFP-c, dFP-c and FP-d.

As the C/EBP family binding site in FP-d is similar to that in pFP-c, while the site in dFP-c is similar to that in FP-a (figure **16.1**), the following discussion is limited to FP-a and pFP-c.

In EMSA studies using nuclear extracts from 5 hr LPS-stimulated RAW 264.7 cells, complex 1/a1 is formed in equivalent abundance with probes FP-a and pFP-c. However, 25-fold excess of pFP-c competitor competes for this complex formed with FP-a probe, while 200-fold excess of FP-a competitor does not fully compete for this complex formed with pFP-c probe; thus, pFP-c binds complex 1/a1 with ~10-fold higher affinity than FP-a. Furthermore, 25-fold excess of pFP-c competitor abolishes complexes 2/a2 and 3/a3 formed with pFP-c probe, but does not fully compete for complex 1/a1 formed with this probe (figure **11.4b**); thus, of complexes 1/a1, 2/a2 and 3/a3, complex 1/a1 has the highest affinity for site pFP-c. As I have suggested that complex 1/a1 contains positive-regulatory C/EBPβ1 protein, this implies that C/EBPβ1 positively regulates MIP1α transcription via the pFP-c and FP-d sites in both unstimulated- and LPS-stimulated RAW 264.7 cells, though as greater levels of complex 1/a1 are formed with nuclear extracts from the LPS stimulated RAW 264.7 cells, a greater level of positive regulation would be expected via these sites in the LPS-stimulated situation.

Site FP-a is homologous to site D of the rat albumin gene promoter (figure **16.1** and Descombes *et al*, 1990). Site D binds C/EBPβ3 homodimers (suggested to be complex 3/a3) with greater affinity than C/EBPβ2/β3 heterodimers (suggested to be complex 2/a2), and the latter with much greater affinity than C/EBPβ2 homodimers (suggested to be complex 1/a1) (Descombes *et al*, 1990).In EMSAs, in a mixture containing equivalent levels of C/EBPβ2 and C/EBPβ3 proteins, approximately equivalent levels of C/EBPβ3 homodimers and C/EBPβ2/β3 heterodimers form with the site D probe, while very low levels of C/EBPβ2 homodimers are formed (Descombes *et al*, 1990). This is equivalent to the EMSA pattern obtained using FP-a probe and unstimulated RAW 264.7 cell nuclear extracts, suggesting that complex

3/a3 has highest affinity-, complex 2/a2 moderate affinity- and complex 1/a1 lowest affinity for site FP-a, though this has not yet been formally tested.

Furthermore, when a 3- to 5-fold excess of C/EBPβ2 over C/EBPβ3 is used in EMSAs with site D probe, no C/EBPβ3 homodimers-, and equivalent levels of C/EBPβ2 homodimers and C/EBPβ2/β3 heterodimers are formed (Descombes *et al*, 1990). This suggests that in RAW 264.7 cells, LPS stimulates the synthesis of C/EBPβ1 (and/or C/EBPβ2 and/or C/EBPδ) protein in preference to C/EBPβ3 protein, so that levels of C/EBPβ1 are ~equivalent to those of C/EBPβ3 protein in unstimulated RAW 264.7 cells, but ~ 3- to 5-fold greater than those of C/EBPβ3 in 5 hr LPS-stimulated RAW 264.7 cells. This would lead to a decrease in C/EBPβ3 homodimers, a large increase in C/EBPβ1 homodimers and a moderate increase in C/EBPb1/b3 heterodimers, as is observed for complexes 3/a3, 1/a1 and 2/a2, respectively. A precedent for this exists, in that C/EBPβ1, C/EBPβ2 and C/EBPβ3 protein levels are differentially modulated in different cell-types by different cell stimuli (Hsu *et al*, 1993).

In co-transfection experiments, using a promoter containing multiple Albumin site D elements, when equivalent levels of C/EBPβ2 and C/EBPβ3 are co-transfected, no promoter activity is observed, whereas co-transfection of 3 to 5-fold more C/EBPβ2 than C/EBPβ3 allows weak promoter activity, though this is nevertheless ~8-fold weaker than that mediated by homodimers of C/EBPβ2 alone. By analogy with the above model, this suggests that C/EBP family proteins may negatively regulate MIP1α promoter activity via FP-a and dFP-c sites in unstimulated RAW 264.7 cells, while mediating weak positive regulation of transcription from these sites in LPS-stimulated RAW 264.7 cells. An added complication is that FP-a can bind PU.1/Spi-B protein at a site overlapping the C/EBP family binding site. In recent preliminary functional transient transfection assays in RAW 264.7 cells (data not presented), point mutation of the PU.1/Spi-B site reduces the activity of the pM-160 MIP1α promoter in unstimulated- but not LPS-stimulated RAW 264.7 cells; this suggests that PU.1/Spi-B positively regulates MIP1α transcription via the FP-a site in unstimulated RAW 264.7 cells, and competes with homodimers of C/EBPβ3 for

binding to FP-a, but is not important for regulation of MIP1α transcription in LPS-stimulated RAW 264.7 cells.

16.3.6 Do cell-specific differences in transcription factor binding to the MIP1α promoter regions containing C/EBP family binding sites contribute to the cell-specificity of MIP1α promoter activity ?

In EMSAs with FP-a or dFP-c probe and MEL cell nuclear extracts, very low levels of complex 1/a1, low levels of complex 2/a2 and high levels of complex 3/a3 are formed. Furthermore, whereas with nuclear extracts from unstimulated RAW 264.7 cells, complexes 1/a1, 2/a2 and 3/a3 are formed in equivalent amounts with pFP-c probe (FP-d probe is discussed separately below), much greater levels of complex 3/a3 than complexes 2/a2 and 3/a3 are formed with pFP-c and MEL cell nuclear extracts. As complex 3/a3 is proposed to contain C/EBPβ3 homodimers, this suggests that C/EBPβ3 negatively regulates the MIP1α promoter via sites pFP-c and dFP-c in MEL cells.

As MEL cells also contain PU.1 and Spi-B proteins (Goebel *et al*, 1990; Moreau-Gachelin *et al*, 1989; Ray *et al*, 1992), this suggests that PU.1/Spi-B competes with C/EBPβ3 for binding to the FP-a site in MEL cells, and as PU.1/Spi-B appear to contribute to MIP1α promoter activity in unstimulated RAW 264.7 cells, this suggests the same may be true in MEL cells. However, PU.1 protein can be phosphorylated at serine 148 by casein kinase II, which does not affect the mobility of PU.1 in EMSAs, but does affect the ability of PU.1 to form protein-protein interactions (Pogubala *et al*, 1993). PU.1 recruits a second factor, NF-EM5, to an adjacent site in the immunoglobulin κ 3' enhancer, and DNA binding by NF-EM5 requires protein-protein interaction with PU.1 and specific DNA contacts (Pongubala *et al*, 1992; Pongubala *et al*, 1993). The PU.1-NF-EM5 complex strongly activates transcription, whereas if PU.1 is mutated at serine 148, although it still binds to its cognate DNA sequence, NF-EM5 is not recruited to the κ 3' enhancer , and no transcriptional activation is observed (Pongubala *et al*, 1993). Hence, it is possible that, depending on the phosphorylation status of PU.1, it is able to recruit other transcription factor(s) to the MIP1α proximal promoter. Given the position of the

PU.1 binding site, such recruitment might involve protein(s) binding to the FP-a CK-1 element or the FP-b Rel family binding site. Furthermore, absence of a CK-1 binding protein-, or lack of PU.1 phosphorylation at serine 148- in MEL cells, but presence of these in RAW 264.7 cells, could contribute to RAW 264.7 cell-specific transcriptional activation of MIP1α transcription, via the FP-a PU.1 binding site.

In EMSAs with FP-a or dFP-c probe and STO cell nuclear extracts, very low levels of complex 1/a1 are formed, while ~equivalent levels of complexes 2/a2 and 3/a3 are formed. Furthermore, STO cells do not contain PU.1 or Spi-B proteins, suggesting that MIP1α transcription is negatively regulated via the FP-a and dFP-c site in STO cells. However, high levels of complex 1/a1 are formed with STO nuclear extracts and pFP-c probe, and this complex is formed with much greater affinity than complexes 2/a2 and 3/a3. This suggests that C/EBPβ1 and/or C/EBPβ2 and/or C/EBPδ can positively regulate transcription via the pFP-c site in STO cells.

MIP1α promoter sites FP-d and pFP-c both contain an AGP/EBP A element-like C/EBP-family binding site (figure **16.1**), and both sites form a similar EMSA binding pattern of C/EBP-related complexes 1/a1, 2/a2 and 3/a3 with nuclear extracts from unstimulated- or LPS-stimulated RAW 264.7 cells. Hence, it is likely that, in terms of C/EBP family proteins, the above discussion involving the pFP-c probe also applies to the FP-d. However, this cannot be confirmed, because nuclear extracts from MEL- and STO cells contain abundant non-C/EBP related proteins, that bind to the FP-d probe, and some of which migrate in EMSAs at the same rate as complex 1/a1.

The FP-d sequence, in addition to containing a weak C/EBP-family binding homology, contains a "CK-2" motif (figure **16.2**), a sequence which is conserved in the promoters of several cytokine genes (Nimer *et al*, 1988). Specific transcription factor binding to CK-2 motifs has not been investigated, but as a CK-2 motif is also contained in the MIP1α promoter FP-f site (figure **16.2**), which does not bind C/EBP family proteins, this shows that the FP-d CK-2 motif is distinct from its C/EBP family binding site. The FP-d CK-2 motif is perfectly conserved with the similar region of the LD78α gene promoter, and with human and murine GM-CSF gene promoters (figure **16.2** and Nimer *et al*, 1988). The CK-2 region of these promoters is protected

Figure 16.2 Sequence alignment of MIP1α gene promoter sites with documented cytokine gene promoter CK-2 motifs.

Sequence comparisons of documented CK-2 motifs from human and murine GM-CSF gene promoters and the murine IFNγ gene promoter (Nimer *et al*, 1988) with MIP1α promoter site FP-d, the corresponding region of the human MIP1α gene homologue LD78α (figure 11.1; Nakao **et al**, 1990) and MIP1α promoter site FP-f.

Human GM-CSF	TGTGGTCAC
Murine GM-CSF	TGTGGTCAC
IFNγ	atctGTCAC
LD78α	TGTGGTCAC
FP-d	TGTGGTCAC
FP-f	ataGGTCAC

in *in-vitro* footprinting experiments using T-cell or myeloid progenitor cell nuclear extracts, and is contained within a region of positive GM-CSF promoter activity in T-cells (Nimer *et al*, 1988; Nomiyama *et al*, 1993). Interestingly, the CK-2 sequence also contains an estrogen response element (ERE) half-site (GGTCA), and genes containing such ERE half-sites are responsive to estrogen in certain cell types (Kato *et al*, 1992).

It cannot yet be excluded that the unknown abundant complexes that form with FP-d probe and STO- and MEL cell nuclear extracts in EMSAs are involved in negative regulation of MIP1α promoter constructs transiently transfected into STO or MEL cells. As discussed above for the pFP-c site, it is likely that negatively-acting C/EBP proteins bind to FP-d in MEL cells, but that positively acting C/EBP proteins bind to FP-d in STO cells. Hence, the potential negative-regulatory effects of the described abundant FP-d-binding complexes are theoretically more important in STO cells than in MEL cells. The possibility that FP-d acts as a negative promoter element in transiently transfected STO and MEL cells could easily be tested, as MIP1α mutant promoter constructs pM-350dm2 and pM-350dm3 (figures **13.1** and **16.6** and Table **6.2**) both contain specific mutations of the FP-d TGTGGTCAC CK-2 motif.

As LPS stimulation induces PF4 family JE and KC mRNAs in Balb/C 3T3 fibroblast cells (Tannenbaum *et al*, 1990), and many cell types express cell surface LPS receptors (Chen *et al*, 1989), this suggests that STO fibroblasts and MEL cells may be responsive to LPS. However, no change in C/EBP-related complex formation in EMSAs was observed upon LPS stimulation of STO- or MEL cells (data not shown). Although no information is available concerning LPS-induced signal transduction in fibroblasts or MEL cells, presumably LPS does not activate signal pathway(s) required to alter the DNA binding of C/EBPβ or C/EBPδ proteins in these cell types.

There is heterogeneity in the expression of C/EBP family proteins between different fibroblast populations. For example, 3T3 L1 cells contain undetectable levels of C/EBPα, C/EBPβ or C/EBPδ proteins prior to differentiation (Cao *et al*, 1991), and this correlates with my EMSA studies using FP-a probe, which failed to

detect any C/EBP-related protein binding using Swiss 3T3 cell nuclear extracts. In contrast to 3T3 cells, both L- and L929 fibroblasts contain detectable levels of C/EBPβ and C/EBPδ, but not of C/EBPα (Roman *et al*, 1990; Akira *et al*, 1990; Chang *et al*, 1990) protein. This correlates with my EMSA data using STO cell nuclear extracts, which suggests that, like RAW 264.7 cells, STO cells contain both C/EBPβ and C/EBPδ proteins. As my data suggests that these proteins are involved in the regulation of MIP1α gene transcription, it therefore also suggests that certain fibroblast cell types may have the potential to express the MIP1α gene, while others do not. This may explain why PMA treatment of primary fibroblasts is reported to induce the MIP1α gene, while the same treatment of Swiss 3T3 cells does not (Nakao *et al*, 1990; M.Plumb, personal communication), as unless PMA treatment of Swiss 3T3 cells induces C/EBPβ and/or C/EBPδ proteins, it would be predicted that the MIP1α gene could not be expressed in these cells.

16.3.7 Post-translational modification of C/EBPβ: possible contribution to LPS-inducibility- and cell-specificity of MIP1α gene expression.

LPS treatment of RAW 264.7 cells up-regulates the activity of the MIP1α pM-160 promoter, and I have proposed that this induction is mediated at least in part by C/EBPβ1 and/or C/EBPβ2 and/or C/EBPδ proteins (henceforth termed C/EBPβ1/2/δ) contained in complex 1/a1 formed with probes FP-a, pFP-c, dFP-c and FP-d. However, while no changes in the binding of C/EBP-related complexes to probe FP-a are detectable until 2-4 hours after the initiation of LPS stimulation, MIP1α mRNA is induced within 30 minutes of the initiation of LPS treatment of RAW 264.7 cells. Possible reasons for this difference in kinetics are: 1) LPS stimulation of RAW 264.7 cells leads to rapid stabilization of MIP1α mRNA, while LPS-induced transcriptional up-regulation is comparatively delayed; 2) LPS-induced binding of NF-κB to site FP-b mediates the earliest transcriptional response of the MIP1α promoter to LPS stimulation of RAW 264.7 cells (see later discussion), while C/EBP family proteins mediate a slower response; 3) C/EBPβ1/2/δ protein present in unstimulated RAW 264.7 cells is rapidly post-translationally modified upon LPS stimulation of the cells, so that over the first 2 hours of LPS stimulation, post-translationally modified

C/EBPβ1/2/δ protein up-regulates MIP1α gene transcription, whereas after 2-4 hours of LPS stimulation, newly synthesized/available C/EBPβ1/2/δ protein then contributes to the LPS-induced up-regulation of MIP1α gene transcription, concomittant with the observed increase in formation of complex 1/a1 in EMSAs.

Evidence suggests that C/EBPβ is regulated largely at the post-transcriptional- (as discussed above) and post-translational levels, while C/EBPδ is regulated at the transcriptional level (Descombes *et al*, 1990; Hsu *et al*, 1993; Ramji *et al*, 1993). Thus, in addition to differential regulation of the relative cellular abundance of C/EBPβ1, C/EBPβ2 and C/EBPβ3 proteins by different signal transduction pathways (Hsu *et al*, 1993), the transactivation potential of C/EBPβ1/2 is enhanced by PKC-induced phosphorylation of Ser 105 in its activation domain, without concommittant increased DNA binding activity (Trautwein *et al*, 1993). Further, a Ras-dependent phosphorylation of Thr 235, next to the DNA binding domain, is essential for trans-activating activity by C/EBPβ1/2 (Nakajima *et al*, 1993). C/EBPβ is also phosphorylated at serine 276 within the leucine zipper, in a calcium-dependent manner by a calcium-calmodulin-dependent protein kinase (Wegner *et al*, 1992). This phosphorylation stimulates the transactivation potential of C/EBPβ1/2 without altering its DNA binding affinity, and is speculated to allow C/EBPβ to alter its heterodimerization partner, by allowing its release from a non-productive dimerization, for example CHOP (Ron *et al*, 1992; Wegner *et al*, 1992) or C/EBPβ3. As outlined in chapter **4**, all of these described C/EBPβ phosphorylation events could potentially be induced by LPS treatment of macrophages.

The cell-specificity of the described post-translational modifications of the transactivating potential of C/EBPβ1/2 has not yet been examined. However, as the described post-translational modifications of C/EBPβ do not appear to affect its DNA binding activity, it is possible that complex 1/a1 formed with pFP-c probe and STO cell nuclear extracts contains C/EBPβ1/2 protein that lacks transactivation potential due to lack of one or more of the phosphorylations described above.

A model depicting the proposed contributions that would be made by C/EBP- and ETS-family proteins to the regulation of transcription of MIP1α promoter/ hGH gene

constructs transiently transfected into unstimulated RAW 264.7-, MEL, STO- and serum-stimulated Swiss 3T3 cells is shown in figure **16.3.**

16.3.8 Does the delayed increase in formation of complex 1/a1 (and 2/a2) with MIP1α promoter C/EBP family binding sites necessarily correlate with positive regulation of the gene ?

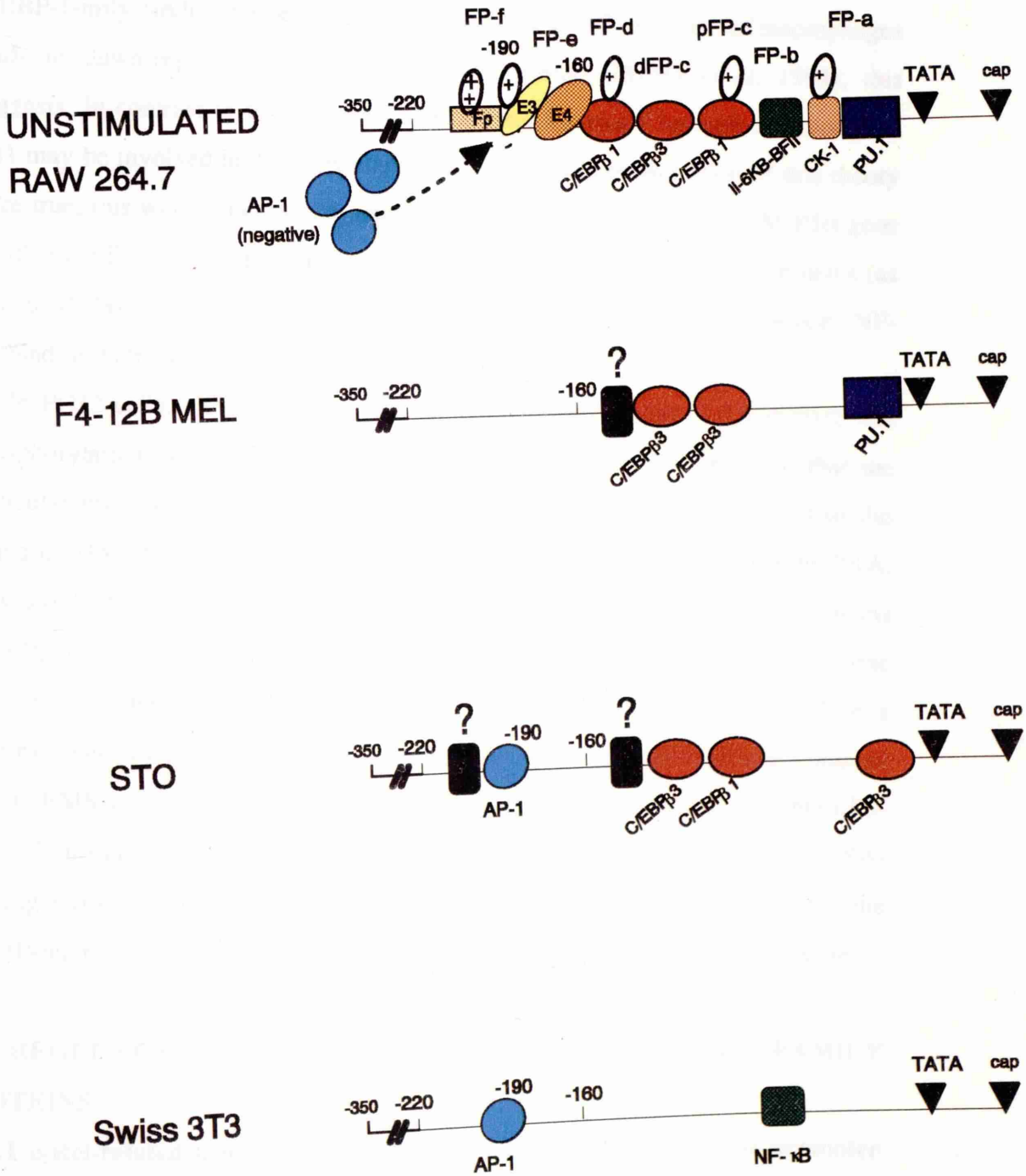
LPS functionally activates macrophages, in the process stimulating the synthesis and release of a range of proteins, including inflammatory cytokines and prostaglandins (Rappolee *et al*, 1989; Chen *et al*, 1989). The prostaglandins feed back onto the macrophage cell-surface, stimulating an increase in the levels of intracellular cAMP, which activates PKA leading to cellular deactivation and attenuation of the production of a sub-population of the LPS-induced inflammtory cytokines (Kunkel *et al*, 1988; Okonogi *et al*, 1991). For example, exogenously-added PGE2 suppresses the LPS-induced production of a number of inflammatory cytokines by macrophages including TNFα and, at least at the mRNA level, MIP1α (Kunkel *et al*, 1988; Martin *et al*, 1991; Okonogi *et al*, 1991).

As outlined in chapter **4**, LPS-mediated signal transduction in macrophages may at least partially be mediated by stimulation of a Gi protein that inhibits adenylate cyclase (Chen *et al*, 1989; Okonogi *et al*, 1991), and LPS pretreatment of macrophages also inhibits the PGE2-mediated up-regulation of cAMP levels (Okonogi *et al*, 1991). This suggests that PKA is not involved in the rapid induction of MIP1α gene transcription in RAW 264.7 cells by LPS. However, assuming RAW 264.7 cells respond to LPS similarly to peritoneal macrophages, a peak of LPS-induced prostaglandin synthesis after ~4 hrs stimulation would be expected to feed back onto the RAW 264.7 cells, thereby down-regulating MIP1α gene expression (Kunkel *et al*, 1988; Martin *et al*, 1991).

cAMP treatment of rat PC12 cells leads to the rapid phosphorylation of cytoplasmic C/EBPβ1/2 protein, which translocates to the nucleus and modulates gene transcription (Metz *et al*, 1991). If a similar mechanism existed in RAW 264.7 cells, PKA-mediated increased DNA binding of C/EBPβ1/2 would be expected after 2-4 hours of LPS stimulation. This is exactly what is observed, assuming that

Figure 16.3: Proposed cell-specific *in-vivo* binding of transcription factors to the MIP1α gene promoter.

Schematic diagram of the MIP1α gene promoter, showing the proposed cell-specific differences in transcription factors regulating transcription of transiently-transfected chimaeric MIP1α promoter/ hGH gene constructs in unstimulated RAW 264.7 cells, F4-12B MEL cells and STO- Swiss 3T3 fibroblasts, as shown. The MIP1α gene TATA box and cap site are shown, and numbers above the promoter denote distance (in bps) from the cap site. Transcription factors are denoted by coloured rectangles, circles and ovals, and bind to MIP1α promoter sites FP-a, FP-b, pFP-c, dFP-c, FP-d, FP-e and FP-f, as shown. An open oval touching a transcription factor represents the proposed positive-regulatory transcriptional activation potential of that factor, and the relative strength of that activation potential is denoted by the number of "+" symbols in the open oval. Transcription factors lacking open ovals are proposed to lack transcriptional activation potential. C/EBPβ1 denotes homo- or heterodimers of C/EBPβ1 and/or C/EBPβ2 and/or C/EBPδ and C/EBPβ3 denotes homodimers of C/EBPβ3, as described in the text. "?" denotes unknown transcription factor with unknown positive/negative regulatory activity. UNSTIMULATED RAW 264.7 CELLS: dotted arrow pointing to dotted circle denotes proposed negative-regulatory transcription factor AP-1 competing for binding to a DNA site occluded by proposed positive-regulatory DNA-bound transcription factors E3 and Fp, as described in the text. STO CELLS: C/EBPβ1 (and/or C/EBPβ2 and/or C/EBPδ, as described above) is proposed to lack post-translational modification, resulting in lack of positive-regulatory activity, as shown and described in the text.


UNSTIMULATED
RAW 264.7
AP-1
(negative)
-350
-220
FP-f
-190
FP-e
FP-d
-160
dFP-c
pFP-c
FP-b
FP-a
TATA
cap
Fp
E3
E4
C/EBPβ 1
C/EBPβ3
C/EBPβ 1
Il-6KB-BFII
CK-1
PU.1
F4-12B MEL
?
C/EBPβ3
C/EBPβ3
PU.1
STO
AP-1
C/EBPβ3
C/EBPβ 1
C/EBPβ3
Swiss 3T3
NF- κB

C/EBPβ1/2 is contained in complex 1/a1 formed in EMSAs with MIP1α promoter C/EBP-family binding probes. Furthermore, because PGE2 treatment of macrophages leads to down-regulation of MIP1α gene expression (Martin *et al*, 1991), this suggests, in contrast to the earlier discussion, that increased formation of complex 1/a1 may be involved in down-regulation of MIP1α gene transcription. If this theory were true, this would suggest that rapid transcriptional induction of the MIP1α gene is either mediated by rapidly post-translationally modified C/EBP family proteins (as described above), or by other transcription factors, for example by LPS-induced NF-κB binding to the FP-b site.

In PC12 cells, although cAMP induces the phosphorylation of C/EBPβ1/2, the phosphorylation is not directly mediated by PKA (Metz *et al*, 1991), so that the particular protein kinase involved may not be present in macrophages, so that the increased DNA binding of complex 1/a1 in response to LPS is *not* mediated by PKA. This would be consistent with my previous proposal that complex 1/a1 contains C/EBPβ1/2 and positively regulates MIP1α gene transcription. In order to test these different hypotheses, I would investigate whether stimulation of RAW 264.7 cells with exogenously-added cAMP or prostaglandins induces the formation of complex 1/a1 in EMSAs with probe FP-a. However, at present, I favour a model wherein LPS-induced prostaglandins induce the synthesis of the negative-regulatory non-DNA-binding C/EBP family protein CHOP in RAW 264.7 cells, which attenuates the C/EBP-related complex 1/a1 up-regulation of MIP1α gene transcription (see below).

16.4 REGULATION OF MIP1α GENE TRANSCRIPTION BY REL-FAMILY PROTEINS.

16.4.1 c-Rel-related transcription factor binding to MIP1α proximal promoter site FP-b in EMSAs.

In the following paragraphs, I discuss evidence for a model in which in a negative-regulatory c-Rel protein-containing transcription factor complex binds to the MIP1α promoter FP-b site in unstimulated RAW 264.7 cells, contributing to keeping

the promoter in a repressed but poised conformation, until being replaced by positive-regulatory NF-κB upon cell stimulation.

In EMSA studies using nuclear extracts from unstimulated- or LPS-stimulated RAW 264.7 cells and FP-b probe, complex b1 is constitutively formed, while complex b2 is LPS-inducible. As complex b2 cross-competes with complex K2 formed with κBpd probe, and formation of both b2 and K2 is LPS-inducible and sensitive to GTP, this suggests that complex b2 contains NF-κB.

Although there is only limited sequence homology between κBpd and FP-b sequences, this homology is part of a larger region of homology between FP-b and other gene regulatory elements which have been demonstrated to bind Rel family proteins, such as the IFNγ C3 intronic enhancer element of the human IFNγ gene (IFNγ C3), the *c-myc* gene internal regulatory element (*c-myc* IRE), which binds NF-κB in an LPS-responsive and GTP-sensitive manner, and the NF-κB binding site of the IL-6 gene promoter (IL-6κB) (figure **16.4**) (Kessler *et al*, 1992; Nakayama *et al*, 1992; Sica *et al*, 1992). Although I have not investigated LPS induction of NF-κB over a timecourse, others have demonstrated that nuclear NF-κB DNA binding activity is significantly induced in RAW 264.7 cells treated with LPS for 30 minutes (Vincenti *et al*, 1992). Thus, NF-κB binding to FP-b may be involved in the rapid transcriptional induction of MIP1α transcription in RAW 264.7 cells in response to LPS.

Nuclear NF-κB DNA binding activity is rapidly induced upon serum stimulation of-, but is undetectable in continuously proliferating- NIH 3T3 fibroblasts (Baldwin, Jr. *et al*, 1991). Similarly, my data shows that in EMSAs using FP-b probe and nuclear extracts from continuously proliferating STO (fibroblasts)- and MEL cells, complex b2 was not formed. I also did not detect complex b2 formation with FP-b probe in EMSAs using nuclear extracts from LPS-stimulated STO- or MEL cells, suggesting that, although these cells may express LPS receptors and contain NF-κB, the signal pathway(s) that activate NF-kB in these cells are not activated by LPS.

Formation of complex b1 with FP-b probe is similar with either unstimulated- or LPS-stimulated RAW 264.7 cell nuclear extracts. b1 formation is GTP-sensitive and

Figure 16.4: Sequence alignment of the MIP1α promoter site FP-b and NF-κB/ c-Rel binding sites.

IFNγ-C3, a c-Rel binding site in the intronic enhancer of the human IFNγ gene; IL-6κB, The NF-κB and c-Rel binding site of the IL-6 gene promoter; κBpd, the palindromic NF-κB binding site used in these studies; c-myc IRE, the NF-κB binding site of the c-myc gene internal regulatory element; FP-a, MIP1α promoter site FP-a (see figure **11.1**).

FP-b (c)	CTTAAAATTTTCCCTCCTCAC
IFNγ-C3	gAATTTTCCa
IL-6κB	ggATTTTCCC
κBpd	gggaaTTCCC
c-myc IRE	AAAaaTTCCCT
FP-a	tccaTTTCCt

is partially competed by κBpd competitor, suggesting that b1 contains NF-κB-related protein(s). The IFNγ C3 element binds c-Rel homodimers but not p50 homodimers in EMSAs, and the sequence-specific binding of c-Rel homodimers depends on those bases which are conserved between IFNγ C3 and FP-b (Sica *et al*, 1992) (figure **16.4**). Furthermore, as the FP-b-homologous IL-6κB element (figure **16.4**) binds c-Rel homodimers with high affinity, p50/c-Rel heterodimers (which co-migrate in EMSAs with p50/p65 heterodimers) with lower affinity, and does not bind p50 homodimers (Nakayama *et al*, 1992), this suggests FP-b can bind c-Rel.

In addition, like FP-b, in EMSA studies IL-6κB forms an inducible NF-κB complex and a constitutively-formed slow-migrating complex that is weakly competed by "standard" NF-κB recognition sequences (Nakayama *et al*, 1992). This factor, termed IL-6κB-BFII, is present in lymphoid- and monocytic cell lines, but little or none is detectable in non-hemopoietic cell types (Nakayama *et al*, 1992). IL-6κB-BFII, which migrates much more slowly than c-Rel homodimers in EMSAs, contains c-Rel- but not NF-κB p50 protein, consistent with the high levels of c-Rel present in lymphoid cells and lack of c-Rel detection in non-hemopoietic cell types (Nakayama *et al*, 1992; Grumont *et al*, 1990). These data suggest that FP-b complex b1 is a c-Rel-containing IL-6κB-BFII-related complex. As would be expected if complex b1 is IL-6κB-BFII, complex b1 is not formed in EMSAs with FP-b probe and nuclear extracts from STO- or MEL cells.

Interestingly, though IL-6κB binds both IL-6κB-BFII and TNFα-inducible NF-κB in EMSAs using T cell nuclear extracts, unlike standard NF-κB binding sites, IL-6κB does not function as a positive TNFα-responsive promoter element in T cells (Nakayama *et al*, 1992). The *in-vivo* TNFα-responsiveness of several κB sites inversely correlates with their ability to bind IL-6κB-BFII *in-vitro*, suggesting that the IL-6κB-BFII complex negatively regulates promoter activity, and that the relative affinity of a particular κB site for IL-6κB-BFII and NF-κB determines the function of that element (Nakayama *et al*, 1992).

The p50 component of NF-κB is apparently constitutively nuclear in macrophages, while p65 is cytoplasmic until the macrophages are activated

(Baeuerle, 1991; Kaufman et al, 1992). As p50 has only a weak activation domain, it is believed to bind to the promoters of NF-κB-regulated genes in unstimulated cells, contributing to keeping those genes in a repressed but "poised" state (Bours *et al*, 1993). As the κBpd competitor used in these studies, (but not IFNγ C3 or IL-6κB), binds $p50_2$ *in-vitro* (Nakayama *et al*, 1992; Ballard *et al*, 1990; Sica *et al*, 1992), this suggests that in EMSAs using nuclear extracts from RAW 264.7 cells, κBpd probe should bind $p50_2$ while FP-b should not, so that the faster-migrating complex obtained with κBpd probe that is competed by κBpd- but not by FP-b competitor (figure **11.10b**), may contain $p50_2$.

Although FP-b apparently does not bind $p50_2$, it does appear to bind an IL-6κB-BFII-related complex present in RAW 264.7 cell nuclear extracts (see above discussion). As IL-6κB-BFII acts as a negative regulator of κB sites in T cells (Nakayama *et al*, 1992), this suggests that, in a similar manner to $p50_2$ binding at standard κB sites, IL-6κB-BFII could contribute to a repressed but poised state of the MIP1α promoter in unstimulated RAW 264.7 cells. Upon LPS stimulation of RAW 264.7 cells, abundant nuclear NF-κB would compete with IL-6κB-BFII for binding to the FP-b site of the MIP1α promoter. Interestingly, my data suggests a mechanism by which binding of IL-6κB-BFII to FP-b would be favoured in unstimulated RAW 264.7 cells, while binding of NF-κB would be favoured upon LPS stimulation, as formation of complexes b1 (IL-6κB-BFII) and b2 (NF-κB) is differentially favoured in different REDOX conditions; this is consistent with the data of others, which shows that release of reactive oxygen intermediates is required for activation of NF-κB *in-vivo*, while binding of NF-κB to κB sites *in-vitro* is REDOX sensitive (Baeuerle et al, 1991; Grimm et al, 1993).

A model depicting the proposed contributions that would be made by IL-6κB-BFII and NF-κB to the regulation of transcription of MIP1a promoter/ hGH gene constructs transiently transfected into unstimulated RAW 264.7-, MEL, STO- and serum-stimulated Swiss 3T3 cells is shown in figure **16.3**.

16.4.2 IL-6κB-BFII and the cell-specific expression of the endogenous MIP1α gene.

STO and MEL cells do not contain nuclear proteins that bind MIP1α promoter site FP-b, while unstimulated RAW 264.7 cells contain an FP-b-binding nuclear IL-6κB-BFII-like complex. It is therefore possible that IL-6κB-BFII may be part of the "jigsaw" of transcription factors (see chapter 3) that are required *in-vivo* for the establishment of an open chromatin conformation over the MIP1α promoter in macrophages. Furthermore, it is possible that though STO- and MEL cells contain nuclear C/EBP- and/or ETS-family proteins, which bind *in-vitro* to sites FP-a, pFP-c, dFP-c and FP-d, the absence in these cell-types of an FP-b-binding transcription factor may mean that this "jigsaw" cannot form, resulting in the detected nucleosome binding (DNase1-resistance) to the MIP1α promoter in these cell types, and lack of endogenous MIP1α gene transcription.

16.4.3 Protein-protein interactions of Rel family proteins: what proteins are present in the IL-6κB-BFII complex ?

IL-6κB-BFII contains c-Rel, but not p50 or the p105 precursor of p50 (Nakayama *et al*, 1992); it has not been tested for the presence of other Rel family members, though c-Rel is able to form heterodimers with all other Rel family members (Nolan *et al*, 1992; Grimm *et al*, 1993), thus allowing for this possibility in the IL-6κB-BFII complex.

C/EBP- and Rel-family proteins can functionally and physically interact (Stein *et al*, 1993), but while Rel-family proteins enhance the specific *in-vitro* DNA binding of C/EBP family proteins, and stimulate C/EBP-family mediated transcription from C/EBP family DNA binding sites, C/EBPα, -β and -δ repress transcription mediated by Rel family proteins from a κB DNA motif (Stein *et al*, 1993). As IL-6κB-BFII represses transcription from certain κB motifs, this might suggest that IL-6κB-BFII contains C/EBP family members. However, two arguments against this are: a) In EMSAs, pFP-c competitor, which binds C/EBP family proteins with high affinity, does not compete for formation of complex b1 (putative IL-6κB-BFII) with FP-b probe; b) Depending on its source, recombinant C/EBPβ protein inhibits the DNA binding of NF-κB p65 and p50 proteins to κB motifs in EMSAs (Stein *et al*, 1993). However, the possibility that C/EBPβ- or -δ proteins may not disrupt high-affinity c-

Rel protein binding to κB motifs such as that in FP-b has not been tested. The promoters of the MIP1α gene and that of other inflammatory cytokines contain adjacent C/EBP family and Rel family binding sites, and the effects of proteins of these two families on each-others binding *in-vitro* to a template containing recognition sequences for both families has not been fully tested. An interesting possibility is that NF-κB-BFII contains C/EBP family proteins, and is a means of sequestering these proteins to a "poised" promoter; in this scenario, displacement of NF-kB-BFII from the MIP1α promoter FP-b site by LPS-induced NF-κB would allow unhindered access of the adjacent C/EBP family binding sites to the C/EBP family proteins, without competition from the FP-b site.

In keeping with this, preliminary data show co-operative binding of C/EBP and NF-κB (sic) to adjacent sites on the IL-8 gene promoter, resulting in a dose-dependent synergistic activation of transcription (Stein *et al*, 1993). In LPS-stimulated RAW 264.7 cells, nuclear NF-κB DNA binding activity is rapidly induced within 30 minutes (Kaufman *et al*, 1992), at which time I have proposed that the FP-a site, adjacent to FP-b, binds predominantly negative-regulatory C/EBPβ3 homodimers, while the adjacent pFP-c site binds positive-regulatory C/EBPβ1/2/δ proteins, suggesting that rapid LPS-induced synergistic activation of MIP1α transcription may occur between NF-κB bound to FP-b and C/EBP family proteins bound to pFP-c.

16.5 POSSIBLE INVOLVEMENT OF THE FP-a SITE ISRE IN LPS-RESPONSIVENESS OF THE MIP1α PROMOTER.

16.5.1 Evidence for specific transcription factor binding to the FP-a site ISRE.

In addition to complexes a1 to a4, containing C/EBP- and ETS-family proteins, faster-migrating complexes are formed in EMSAs using FP-a probe. The diffuse complex formed using RAW 264.7 cell nuclear extracts, and which migrates between complexes a4 and a5, is competed by FP-a and PU.1 competitors (figure **11.2b**), suggesting it may be ETS-related. It is also competed by κBpd competitor (figure

11.2b), which may be explained by the fact that the κBpd sequence contains a GGAA core sequence, recognised by ETS family proteins (Karim *et al*, 1990).

A second faster-migrating complex, complex a5, is not competed by C/EBP family-binding competitors (figure **11.2b**), so it does not contain C/EBP family members or their degradatory products. Further, as formation of complex a5 declines upon LPS stimulation of RAW 264.7 cells (just visible in figure **11.4a**), while formation of PU.1/Spi-B-containing complex a4 is unaffected by LPS stimulation, complex a5 does not contain PU.1/Spi-B and/or their degradatory products, even though formation of complex a5 with FP-a probe is competed by PU.1 competitor. This suggests that the FP-a and PU.1 probes contain a transcription factor binding site homology distinct from the PU.1/Spi-B binding site.

In addition to C/EBP- and ETS-family binding sites, the FP-a site contains an ISRE homology (Schindler *et al*, 1992; Fu, 1992). The ISRE can bind the ISGF3 transcription factor upon cell stimulation with IFNα/β, and prior to cell stimulation, the ISGF3γ component of this complex can bind separately to the ISRE with low affinity (Schindler *et al*, 1992; Fu, 1992). The ISGF3γ protein is a member of a family of transcription factors, termed the IRF family, which all share DNA binding specificity related to but distinct from that of ISGF3γ, in that ISGF3γ interacts with the entire ISRE sequence, but fails to bind a shorter DNA sequence called PRDI, while the other family members require only the core ISRE sequence or the short PRDI sequence in order to bind DNA (Veals et al, 1992). Like the a5 complex formed with FP-a, IRF family protein-probe complexes migrate rapidly in EMSAs (Schindler *et al*, 1992; Fu, 1992; Veals *et al*, 1992), and of the oligonucleotide probes used in this study, only the FP-a and PU.1 probes contain either an ISRE (FP-a) or a potential PRDI sequence (PU.1: GAAAGa c.f wild-type PRDI GAAAGT), suggesting that complex a5 formed with FP-a probe and competed by PU.1 competitor may contain IRF family protein(s).

Of the IRF family, IRF-1 mRNA is (slightly) induced in by LPS stimulation of RAW 264.7 cells (Farber, 1992), and as complex a5 is reduced by LPS treatment of RAW 264.7 cells, this suggests complex a5 contained in RAW 264.7 cell nuclear

extracts is not IRF-1. IRF-2 mRNA levels are also reported to be modulated by LPS treatment of cells (Harada *et al*, 1989) (the reference does not mention cell type or whether the modulation is an induction or a repression), suggesting that RAW 264.7 cell complex a5 may be IRF-2. Another IRF family member, ICSBP, is hemopoietic cell-specifically-expressed (Weisz *et al*, 1992), and both IRF-2 and ICSBP are negative regulators of transcription (Harada *et al*, 1989; Veals *et al*, 1992; Weisz *et al*, 1992). As LPS stimulation of RAW 264.7 cells leads to a decrease in formation of complex a5, this suggests that a5 may contain IRF-2 or ICSBP.

As complex a5 obtained with FP-a probe and RAW 264.7 cell nuclear extracts is competed by PU.1 competitor, and the PU.1 sequence contains a potential PRDI motif but not an ISRE (Table **6.3**), this suggests RAW 264.7 cell complex a5 is not ISGF3γ. In contrast, complex a5 formed with FP-a probe and STO cell nuclear extracts is not competed by the PU.1 competitor, and as the only IRF family protein that binds to the ISRE (present in FP-a) but not to the PRDI motif (putatively present in PU.1) is ISGF3γ, this suggests that complex a5 obtained with FP-a probe and STO cell nuclear extracts may contain ISGF3γ protein.

16.5.2 The ISRE binds LPS-induced ISGF3 in RAW 264.7 cells.

In LPS-stimulated RAW 264.7 cell nuclear extracts, an LPS-inducible complex was observed in EMSAs using an IP-10 gene ISRE probe (Ohmori *et al*, 1993). The complex was first detectable, and was maximal, at 3 hours after LPS stimulation, was still present at 12 hours, but was undetectable 24 hours after stimulation (Ohmori *et al*, 1993). The same complex was induced by IFNγ, but though the induction was much more rapid, much less of the complex was formed than with LPS stimulation (Ohmori *et al*, 1993). The ISRE was required for IFNγ- but not LPS induction of the IP-10 promoter, though multimerized ISRE motifs were able to confer LPS-responsiveness on a heterologous promoter in RAW 264.7 cells (Ohmori *et al*, 1993). Interestingly, this LPS-responsiveness was blocked by antibody to IFNα/β, and as LPS induces the production of IFNα/β by macrophages, this suggests that functional LPS-responsiveness of the ISRE in RAW 264.7 cells is indirect, and due to LPS-induced IFNα/β feeding back onto the cells (Ohmori *et al*, 1993). As the initial LPS-

responsiveness of the IP-10 promoter in RAW 264.7 cells appears to be conferred by two promoter κB sites, the ISRE may confer a prolonged LPS-responsiveness (Ohmori *et al*, 1993). Together with the evidence that MIP1α promoter site FP-a may bind ISRE-specific transcription factors, this suggests that the FP-a site ISRE may prolong the LPS-induced transcriptional up-regulation of the MIP1α gene in RAW 264.7 cells, initially mediated by C/EBP- (pFP-c and dFP-c) and κB (FP-b) motifs.

16.6 THE MIP1α PROMOTER DISTAL (-160bp to -220 bp) REGULATORY ELEMENT (DRE).

In EMSAs, a slow-migrating complex formed with FP-f probe and RAW 264.7-, STO- and NIH 3T3 cells co-migrated and cross-competed with a complex formed with an AP-1 binding probe, E3-AP-1, but was not competed by a mutated E3-AP-1 competitor containing specific point mutations in the AP-1 binding site. As the formation of this complex with FP-f probe was also enhanced in reducing conditions, characteristic of AP-1 (Abate *et al*, 1990), this suggests the complex contains AP-1.

Although many studies using nuclear extracts from serum stimulated NIH 3T3 cells have shown the presence of c-Fos in AP-1 complexes (Gillespie, 1991), my preliminary antibody studies suggest that the AP-1 complex formed with FP-f probe does not contain c-Fos protein. However, in asynchronously growing NIH 3T3 cells, c-Fos and FosB are expressed at very low levels, and Fra-1 and Fra-2 are the main Fos proteins complexed with c-Jun protein, though in the the first hour after serum stimulation of these cells, AP-1 contains mainly c-Fos and c-Jun proteins (Kovary et al, 1992). Hence, my nuclear extracts from NIH 3T3 cells serum-stimulated for 4 hours- and, by analogy, asynchronously-growing RAW 264.7 cells, would be expected to contain very low levels of c-Fos in AP-1 complexes.

No information is available on the levels of Fos proteins in LPS-stimulated macrophages, though LPS stimulation is known to induce *c-fos* mRNA levels in macrophages (Fujihara *et al*, 1993). However, upon LPS treatment of J774.2 macrophage cells, both cellular levels of JunB protein and observed AP-1 DNA binding to a TRE probe in EMSAs noticeably increased within 10 minutes and

continued to increase over an 8 hour timecourse (Fujihara *et al*, 1993). Examination of the Jun family members present in TRE complexes over the 8 hour timecourse revealed constant levels of c-Jun and JunD proteins, while the levels of Jun B increased steadily over the timecourse (Fujihara *et al*, 1993).

The FP-f probe (figure **11.1**) does not contain a canonical TRE sequence (Hai *et al*, 1991), but in its 3' region it contains a sequence (TGAGACTT) which has exact homology to a region of the human TGFβ1 gene promoter which confers positive TGFβ (and TPA) responsiveness on a reporter gene in a c-Fos and c-Jun protein-dependent manner (Kim *et al*, 1989; Kim *et al*, 1990). Interestingly, the TGAGACTT sequence overlaps the 5' end of site FP-e and the 3' end of site FP-f that were protected in *in-vitro* footprinting experiments (figure **11.1**). This suggests that binding of AP-1 to the TGAGACTT sequence within the context of the MIP1α promoter in unstimulated or 12 hr LPS-stimulated RAW 264.7 cells is occluded by binding of transcription factors to the adjacent FP-e and FP-f sites. However, as I have shown that TGFβ treatment of RAW 264.7 cells down-regulates the levels of MIP1α mRNA, this suggests that AP-1 may bind in a TGFβ-responsive manner to a site overlapping footprinted sites FP-e and FP-f *in-vivo*, thus disrupting binding of transcription factors to the FP-e and FP-f sites, and down-regulating the MIP1α gene at the level of transcription.

AP-1-mediated negative regulation of transcription by TGFβ has previously been observed, via an element called the TGFβ inhibitory element (TIE) (Kerr *et al*, 1990; Kerr *et al*, 1992). However, the TIE element sequence is different to that of both a canonical TRE element and TGFβ-responsive AP-1 binding site described above (Kerr *et al*, 1990; Kim *et al*, 1990). These sequence differences may be important in determining which Fos-, Jun- or ATF-proteins are contained in the AP-1 complexes that bind to these different sites (Hai *et al*, 1991), so that AP-1 binding to these different sites may have different functional consequences; for example, the stromelysin gene promoter contains TIE and TRE elements, both of which bind AP-1, but the TIE element negatively-regulates-, while the TRE element positively-regulates- stromelysin gene transcription (Matrisian *et al*, 1992; Kerr *et al*, 1992).

In my studies, I did not detect an increase in AP-1 binding to the FP-f probe in LPS-stimulated- compared to unstimulated RAW 264.7 cells. This may be because the nuclear extracts used were from 22-hour LPS stimulated RAW 264.7 cells, but it is also possible that different AP-1 complexes in RAW 264.7 cell nuclear extracts form preferentially with the TRE and FP-f probe AP-1 binding sites, rendering the latter unresponsive to LPS treatment of RAW 264.7 cells. Further, it is possible that, of these AP-1 sites, only the TIE element is able to confer negative regulation of transcription in response to TGFβ. These possibilities have yet to be examined.

16.7 SERUM-INDUCIBILITY OF TRANSCRIPTION OF THE MIP1α GENE IN RAW 264.7 CELLS: WHY FRESH SERUM-INDUCED UP-REGULATION OF MIP1α TRANSCRIPTION IS PROLONGED, WHILE LPS-INDUCED TRANSCRIPTIONAL UP-REGULATION IS TRANSIENT.

A puzzling aspect of the study of the induction of MIP1α gene expression in macrophages by LPS and fresh serum is that, depending on the investigator, rapidly-induced MIP1α mRNA can either return to basal levels within 24 hours or remain at significantly-elevated levels for several days (my data; Davetalis et al, 1988; M. Plumb, personal communication; P. Daubersies, personal communication; A. Reid, PhD thesis, 1992). MIP1α is an early-response gene in macrophages, and as such, its expression appears to be controlled at multiple levels, including modulation of the rate of initiation- and elongation of transcription (this thesis) and of the stability of its mRNA (M. Plumb, personal communication; A. Reid, PhD thesis, 1992). Thus there are several possible mechanisms by which the observed differences could occur; however, as yet there is no explanation for these differences. In the following paragraphs, I will present a testable model which addresses the issue of why rapidly induced MIP1α mRNA levels are sometimes rapidly attenuated and sometimes more prolonged.

In my studies of MIP1α RNA levels in RAW 264.7 cells, after replating of cells in cell growth medium containing fresh 10% (v/v) FCS, it was necessary to incubate the cells for 3 days before MIP1α mRNA returned to basal levels, in contrast to LPS

stimulation of RAW 264.7 cells, in which MIP1α mRNA was back to basal levels after 24 hours of stimulation. That this prolonged serum-responsiveness is at least partly at the level of transcription is suggested by the differences between Experiment 1 and Experiment 2 in the functional studies, which suggested that MIP1α promoter element(s) in the FP-e site are more active 2 days after the initiation of serum stimulation than after 3 days. A prolonged transcriptional up-regulation of the MIP1α gene in response to fresh serum stimulation of RAW 264.7 cells is further supported by EMSA studies of C/EBP family protein binding to MIP1α promoter site FP-a; in contrast to LPS-stimulation, in which the proposed positive regulatory complex 1/a1 has returned to ~basal levels after 16 hours of LPS stimulation, complex 1/a1 is at significantly induced levels after 15-24 hours of fresh 10% (v/v) FCS stimulation.

A basis for a transient induction of MIP1α transcription in RAW 264.7 cells by LPS and a prolonged induction by fresh 10% (v/v) FCS may be that treatment of RAW 264.7 cells with LPS is cytostatic, while treatment with 10% FCS is not (Vairo *et al*, 1992). Certain genes are induced upon cellular growth arrest, and one of these is the C/EBP family member CHOP (Fornace *et al*, 1989; Ron *et al*, 1992). CHOP can dimerize with other C/EBP family members, and prevents them from binding DNA, as it has 2 prolines substituted in its basic region (Ron *et al*, 1992). Thus, the transient nature of LPS-up-regulated MIP1α transcription may be explained by the LPS-mediated growth-arrest induction of CHOP in RAW 264.7 cells, which then dimerizes with all of the C/EBP family proteins, including negative-regulatory C/EBPβ3, which bind to FP-a, pFP-c, dFP-c and FP-d, resulting in reduction of binding of all C/EBP related complexes to these sites, as is progressively observed subsequent to 6 hours of LPS stimulation, and attenuation of MIP1α transcription.

In addition to LPS, exogenously-added cAMP is also cytostatic for macrophages (Rock *et al*, 1992), so may also induce CHOP synthesis (Fornace et al, 1989). As discussed earlier, LPS treatment of macrophages stimulates the release of prostaglandins, which feed back on to the cells, increasing the levels of intracellular cAMP (Rappollee *et al*, 1989; Chen *et al*, 1989; Kunkel *et al*, 1988; Okonogi *et al*, 1991); furthermore, exogenously-added prostaglandins down regulate MIP1α gene

expression in macrophages (Martin et al, 1991). Thus, I propose a mechanism whereby LPS stimulates the production of prostaglandins by RAW 264.7 cells, which feed back on to the cells, arresting cell proliferation and inducing synthesis of CHOP. CHOP then attenuates the C/EBPβ1/2/δ-mediated rapidly-induced MIP1a transcription by dimerizing with these proteins (and with C/EBPb3) and thus preventing them from binding to DNA. As 10% (v/v) FCS does not induce cellular growth arrest, the 10% (v/v) FCS induction of positive-regulatory C/EBP family proteins would not be accompanied or followed by induction of CHOP, and thus would allow the prolonged DNA binding of positive-regulatory C/EBP family proteins.

Because RAW 264.7 cells are a permanent tissue-culture cell-line, it may be difficult to consistently arrest their proliferation; this may depend on parameters such as the level of positive serum growth factors present, the concentration of cytostatic agents used (I used very high levels (2 μg/ml) of LPS) and the confluence of the cells. Thus, I propose that differences in these parameters may, by affecting the proliferation status of the cells, differentially induce CHOP levels, which may contribute to whether or not the induction of MIP1α gene expression is transient or more prolonged. In each experimental system used, measurement of CHOP levels and monitoring the levels of C/EBP family proteins binding to the MIP1α promoter would provide evidence for or against ther above theory.

Another potentially significant difference between the LPS- and fresh 10% FCS-modulation of C/EBP family binding to FP-a probe is that while on LPS stimulation complex 3/a3 declines concomittantly with the increase in complexes 1/a1 and 2/a2, though complexes 1/a1 and 2/a2 are markedly increased after 15 hours of FCS stimulation, complex 3/a3 has not declined. In the hypothesis proposed earlier, complex 1/a1 is a positive regulator containing C/EBPβ1/2/δ, while complex 3/a3 contains negative-regulatory C/EBPβ3, and preferential synthesis of C/EBPβ1/2/δ over C/EBPβ3 upon LPS stimulation leads to incorporation of C/EBPβ3 into heterodimers (complex 2/a2) with C/EBPβ1/2/δ. Thus, as complex 3/a3 does not decline upon fresh 10% (v/v) FCS stimulation of RAW 264.7 cells, it appears that

C/EBPβ3 protein synthesis is also stimulated by fresh serum. Further, it is therefore possible that MIP1α promoter sites FP-a and dFP-c confer LPS responsiveness- but do not confer serum responsiveness on the MIP1α gene. However, as outlined earlier, complex 1/a1 has much greater affinity than complexes 2/a2 or 3/a3 for MIP1α promoter site pFP-c (and probably FP-d), so that serum-responsive transcription would be expected to be conferred by sites pFP-c and FP-d.

I have not examined induction of nuclear NF-κB DNA binding in response to fresh FCS stimulation of RAW 264.7 cells. However, it is possible that rapid transcriptional induction of the MIP1α gene by fresh FCS is conferred by NF-κB binding to FP-b and by post-translational modification of C/EBPβ1/2 (as described earlier), while a prolonged transcriptional response to fresh FCS is conferred by prolonged FCS-induced synthesis of C/EBPβ1/2/δ protein, and binding of these to sites pFP-c and FP-d, and by a similarly prolonged FCS-induced synthesis of protein(s) binding to the FP-e site.

Serum-induced up-regulation of MIP1α gene transcription in macrophages appears to be distinct from the serum-induced transcription of many other genes in susceptible cell types, which occurs via transcription factor complexes containing SRF, binding to promoter SRE [$CC(A/T)_6GG$] motif(s) (Treisman, 1990; Treisman, 1992). Further genes contain rapidly serum responsive promoter regions containing DNA binding sites [$CTA(A/T)_4TAG$] for a family of transcription factors called Related to Serum Response Factor (RSRF) proteins (Pollock *et al*, 1991; Han *et al*, 1992). Interestingly, the human MIP1α gene homologue LD78α contains an SRE element at position -672, which is not conserved in the MIP1α promoter (Widmer *et al*, 1991), while the MIP1α promoter contains a potential RSRF binding site, CTATTTcTAG at position -634 (RSRF can bind to sites containing a central C nucleotide) (my observation; Widmer *et al*, 1991; Pollock *et al*, 1991), and this site is not conserved in the LD78α promoter (Nakao *et al*, 1990). However, as the potential RSRF binding site is upstream of the region of the MIP1α promoter that is serum responsive in RAW 264.7 cells, this suggests that this site may be important for

serum- and growth factor responsiveness of the MIP1α promoter in non-macrophage cell types.

Although there is no published data on the involvement of C/EBP family proteins in serum-responsive transcription, the involvement of other non-SRF and -RSRF proteins in serum responsive transcriptional regulation has been both documented and implicated. For example, Rel family genes are induced by serum stimulation of NIH 3T3 cells (Ryseck *et al*, 1992; Baldwin Jr. et al, 1991), and the BBF-1 transcription factor is contained only in cardiac muscle cells that are cultured in medium containing a high concentration of serum (Zhou *et al*, 1993).

16.8 COMPARISON OF FUNCTIONAL LPS- AND SERUM-RESPONSIVENESS OF THE MIP1α PROMOTER CONSTRUCTS IN RAW 264.7 CELLS.

It is evident that LPS and fresh FCS do not alter the activity of MIP1α promoter constructs by the same mechanism, in that all of the promoter constructs are serum-responsive, whereas longer promoter constructs are not LPS responsive. In the following paragraphs, I discuss evidence in support of a model in which fresh FCS and LPS rapidly induce MIP1α gene transcription in RAW 264.7 cells using overlapping but distinct sets of transcription factors.

In a study of the effects of different concentrations of fresh FCS on the activity of the pM-160 promoter in RAW 264.7 cells, it was observed that the serum-stimulated activity was equally maximal in either 5%- or 10% (v/v) fresh FCS, indicating that 5% (v/v) fresh FCS was equally as effective as 10% (v/v) fresh FCS in the induction of serum responsive transcription factors that positively regulate MIP1α gene promoter activity.

The response of the minimal pM-160 MIP1α promoter to LPS stimulation of RAW 264.7 cells was maximal in the presence of fresh 1% (v/v) FCS, even though stimulation with the latter alone had minimal effect on the activity of this promoter. Increasing the amount of fresh serum present, from 1%- to 5%- to 10%- (v/v) led to a progressive decrease in the LPS-responsiveness of the pM-160 promoter, though the

promoter was LPS-responsive at all tested concentrations of fresh FCS. The simplest explanation for these effects may be that LPS and fresh serum stimulation of RAW 264.7 cells lead to the synthesis/ mobilization/ activation/ induction of DNA binding of certain transcription factors in an identical manner, but that LPS stimulation also either activates other different transcription factor(s) or leads to the modification of the transcription factor(s) induced by fresh serum, so that these factor(s) have increased transcriptional activation activity. In support of this idea, evidence discussed above suggests that while sites FP-a, pFP-c, dFP-c and FP-d are LPS-responsive, only pFP-c and FP-d are serum-responsive, and that while site FP-e appears to be serum-responsive, there is no evidence that it is LPS-reponsive. In addition, it is possible that any or all of the described transcription factors could be differentially post-translationally modified in response to fresh FCS-stimulation-compared to LPS-stimulation of RAW 264.7 cells.

As the experiments comparing LPS-regulation of MIP1α transcription in RAW CM compared to medium containing 1%-, 5%- and 10% (v/v) fresh FCS were all conducted using the same transfection conditions ("Experiment 1" conditions, see above), they can be directly compared, so that as the 1%- and 5% (v/v) fresh FCS stimulations were done fewer times than the RAW CM and 10% (v/v) fresh FCS stimulations, they are probably less accurate. Further, as discussed earlier, the pM-160 promoter -fold LPS-stimulation in RAW CM was greater in Experiment 2, when experimental conditions were adjusted to reduce the levels of MIP1α transcription-inducing serum growth factors in the RAW CM, and the LPS-induced transcriptional activity of the pM-160 promoter was similar in the presence of RAW CM or fresh 1% (v/v) FCS once these adjustments had been made (figure **13.3**).

In light of this discussion, it is reasonable to suggest that RAW CM and 1% (v/v) FCS contain similarly low levels of MIP1α transcription-inducing serum growth factors. Stimulation of the RAW 264.7 cells with LPS under these conditions would then be expected to induce the *in-vivo* DNA binding of both the set of transcription factors whose *in-vivo* binding to the MIP1α promoter I have suggested to be uniquely-inducible by LPS (ie. C/EBPβ1/2/δ binding to sites FP-a and dFP-c), and

the set of transcription factors also potentially induced by higher concentrations of fresh FCS (ie. C/EBPβ1/2/δ binding to sites pFP-c and FP-d). In the presence of higher concentrations of fresh FCS, *in-vivo* promoter binding of the latter transcription factors would already be induced, therefore reducing the LPS-responsiveness of the MIP1α promoter, as discussed above. Also, as the kinetics of induction of MIP1α gene transcription by fresh FCS and LPS may be different, different concentrations of fresh FCS might differentially modulate the kinetics of LPS-induced MIP1α gene transcription.

A model summarizing the proposed differences in the transcription factors binding to the MIP1α promoter in unstimulated RAW 264.7 cells, and at different times after LPS- or fresh 10% (v/v) FCS stimulation is shown in figure **16.5** (proteins shown binding to sites FP-e and FP-f in this figure are discussed in section **16.9**, below).

16.9 FUNCTIONAL TRANSIENT TRANSFECTION STUDIES OF POINT-MUTATED MIP1α PROMOTER CONSTRUCTS IN RAW 264.7 CELLS.

Initial functional transient transfection studies of the activities of wild-type MIP1α promoter constructs in RAW 264.7 cells suggested that all of the promoter sequences functional in these cells were between positions +36 bp and -190 bp (chapter **10**). Hence, I inserted point mutations into the pM-190 MIP1α promoter/ hGH gene construct. However, after adjustment of transfection conditions, functional studies with the wild-type MIP1α promoters suggested, in agreement with *in-vitro* footprinting experiments, that a further functional promoter region is present between -190 bp and -220 bp (see chapter **12** overview and figure **13.3**), so that the FP-e and FP-f regions both contain positive-regulatory promoter elements, and I therefore inserted the same point mutations into the pM-350 MIP1α promoter construct, in order to investigate whether the functional outcome of these point mutations was affected by their promoter context.

As *in-vitro* footprinting studies detected transcription factor(s) binding to both the FP-e and FP-f sites, while EMSA studies only detected binding of AP-1 to a site

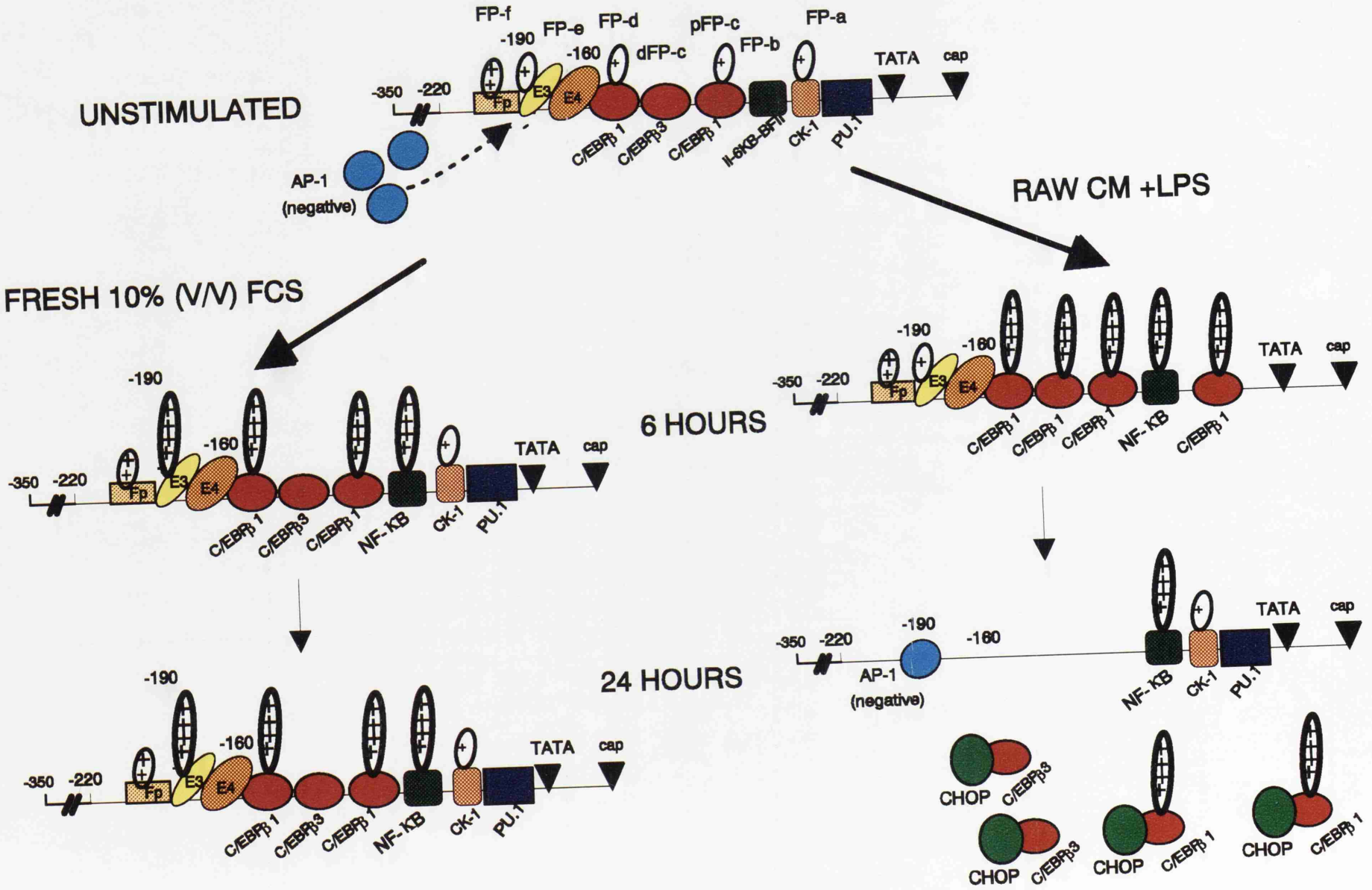

UNSTIMULATED
FP-f
-190
FP-e
FP-d
-160
dFP-c
pFP-c
FP-b
FP-a
TATA
cap
-350
-220
Fp
E3
E4
C/EBPβ 1
C/EBPβ3
C/EBPβ 1
IL-6KB-BF1
CK-1
PU.1
AP-1
(negative)
RAW CM +LPS
FRESH 10% (V/V) FCS
6 HOURS
NF- KB
24 HOURS
CHOP
C/EBPβ3
C/EBPβ 1

Figure 16.5: Proposed transcription factors binding to the MIP1α gene promoter *in-vivo* in RAW 264.7 cells under different cellular conditions.

Schematic diagrams of proposed transcription factors binding to the MIP1α promoter in RAW 264.7 cells in RAW CM (unstimulated), or after stimulation of the cells for 6- or 24 hours in either RAW CM containing 2 μg/ml of LPS or fresh medium containing fresh 10% (v/v) FCS, as shown. The MIP1α gene TATA box and cap site are shown, and numbers above the promoter denote distance (in bps) from the cap site. Transcription factors are denoted by coloured rectangles, circles and ovals, and bind to MIP1α promoter sites FP-a, FP-b, pFP-c, dFP-c, FP-d, FP-e and FP-f, as shown above the unstimulated promoter. An open oval touching a transcription factor represents the proposed positive-regulatory transcriptional activation potential of that factor, and the relative strength of that activation potential is denoted by the number of "+" symbols in the open oval. Transcription factors lacking open ovals are proposed to lack transcriptional activation potential. Promoter-bound transcription factors: C/EBPβ1 denotes homo- or heterodimers of C/EBPβ1 and/or C/EBPβ2 and/or C/EBPδ and C/EBPβ3 denotes homodimers of C/EBPβ3, as described in the text. Free transcription factors: C/EBPβ1 denotes monomers of C/EBPβ1 and/or C/EBPβ2 and/or C/EBPδ and C/EBPβ3 denotes monomers of C/EBPβ3, respectively, dimerized with monomers of negative-regulatory CHOP protein, as shown and described in the text. UNSTIMULATED RAW 264.7 CELLS: dotted arrow pointing to dotted circle denotes proposed negative-regulatory transcription factor AP-1 competing for binding to a DNA site occluded by proposed positive-regulatory DNA-bound transcription factors E3 and Fp, as described in the text.

overlapping FP-e and FP-f, this suggests that transcription factor binding to the FP-e and FP-f regions may require co-operativity between transcription factors binding to these two regions and/or with transcription factors binding to the proximal MIP1α promoter (ie. between +36 bp and -160 bp). The latter possibility is supported by functional studies with pM-350 MIP1α promoters with point mutations in site FP-d (see below).

In RAW 264.7 cells, the FP-d sequence binds C/EBP family proteins, and I have proposed that in both unstimulated- and LPS-stimulated RAW 264.7 cells, this site binds C/EBP-related complex 1/a1 (C/EBPβ1/2/δ), which positively regulates the MIP1α promoter. Because, unlike sites FP-a and dFP-c, site FP-d probably binds complex 1/a1 before and after LPS stimulation, this suggested that FP-d probably contributes more than these other sites to MIP1α gene transcription in unstimulated RAW 264.7 cells.

FP-d site mutations dm1, dm2 and dm3 specifically mutate the proposed C/EBP family binding site, while mutation dm4 does not, and mutant pM-350 promoters containing mutations dm1, dm2 or dm3 have significantly less activity than wild-type pM-350 in unstimulated RAW 264.7 cells, while pM-350dm4 has wild-type activity (figure **16.6**). This confirms the suggestion that FP-d contributes significantly to MIP1α promoter activity in unstimulated RAW 264.7 cells. Furthermore, as the activities of these dm1-, dm2-, and dm3 mutant pM-350 promoters are much more similar to that of wild-type pM-160 than wild-type pM-350 (figure **16.6**), the strong positively-acting promoter elements within the FP-e and FP-f regions of the MIP1α promoter are able to exert very little transcriptional-regulatory effects in these FP-d-mutant promoters. One possible reason for this may be that C/EBP-family proteins interacting with the FP-d site recruit transcription factor(s) to the FP-e site and thence to the FP-f site; this possibility is supported by the *in-vitro* footprinting and EMSA studies, in that sites FP-e and FP-f bind transcription factors when in the context of the whole promoter (footprinting) but not when tested individually (EMSAs). Another possibility is that transcription factors binding to the FP-e and FP-f regions can only communicate with the proximal promoter and transcriptional preinitiation

complex via protein-protein interactions involving C/EBP family proteins interacting with the FP-d site. As there are multiple C/EBP family binding sites in the proximal MIP1α promoter, a combination of the two described mechanisms may occur, in which C/EBP family binding to the FP-d site is required in order for transcription factor(s) to bind to the adjacent FP-e site, or for the latter factors to interact with the proximal promoter-bound transcription factors.

As discussed earlier, binding of negative-regulatory AP-1 to a site overlapping the MIP1α promoter footprinted sites FP-e and FP-f may be occluded *in-vivo* by transcription factors binding to these adjacent sites. However, as JunB protein- and *c-fos* mRNA (Fujihara *et al*, 1993) are induced by LPS stimulation of RAW 264.7 cells, a role for AP-1 in the LPS-response of the MIP1α promoter in RAW 264.7 cells cannot be ruled out. For example, the AP-1 site could be inolved in both positive and negative regulation of MIP1α promoter activity in RAW 264.7 cells. Furthermore, as discussed earlier, it is apparent that different AP-1 complexes are predominant in cells under different conditions (eg. serum stimulation) (Kovary *et al*, 1992), and that different AP-1 DNA binding sites probably selectively bind distinct AP-1 complexes (Hai *et al*, 1991). Hence, during LPS stimulation of RAW 264.7 cells, different AP-1 complexes might become available at different times, and the affinity of these different complexes for the MIP1α promoter AP-1 binding site, and their functional effects, may vary.

The proposed site of AP-1 binding in the FP-f probe is TGAGACTT (homologous to TGFβ1 gene promoter AP-1 binding sites: figure **12.1** and Kim *et al*, 1990); however, in the pM-190 promoter/hGH gene construct, the two bases at the 5' end of the above site are deleted, and replaced by the sequence TT (see Table 6.4, PCR primer pM-190p), so that the pM-190 promoter contains the sequence TTAGACTT, which I assume is still able to bind AP-1. This site is mutated by mutation em1 (figure **16.6**). Mutation em4 introduces a possible second AP-1 binding site (aGAGAGaaT compared to wild-type TGAGAGCTT) into the pM-190 and pM-350 MIP1α promoter sequences (figure **16.6**), and the possible functional consequences of this will be discussed below.

The presence of a perfect inverted repeat motif (ATCTCaGAGAT) in the FP-e footprinted sequence (figure **16.6**) suggests this site may bind a putative dimeric transcription factor, henceforth termed factor E3. Mutation em2 encompasses the distal half-site, and em3 encompasses the proximal half-site and overlaps the distal half-site of the inverted repeat (figure **16.6**). However, in unstimulated RAW 264.7 cells, while promoter pM-190em2 has ~60% of wild-type activity, promoter pM-190em3 has only 3% of wild-type pM-190 promoter activity (figure **16.6**). A possible explanation for this is that factor E3 is recruited to the DNA by a second putative transcription factor, henceforth termed factor E4, binding to the proximal region of the FP-e site (figure **16.6**). The presence of the proximal half-site of the E3 transcription factor binding site directly adjacent to the E4 binding site (as in mutant pM-190em2, figure **16.6**), may be sufficient to allow recruitment of E3 to the DNA by E4, whereas as mutation em3 affects both half-sites of the E3 binding site (figure **16.6**), this may be sufficient to prevent the recruitment of E3 to the DNA by E4 alone.

Precedents for these concepts are the recruitment of NF-EM5 to the κ 3' enhancer by PU.1 (Pongubala *et al*, 1992; Pongubala *et al*, 1993), and the stabilization of DNA binding of estrogen receptors to ERE half-sites by adjacently-bound transcription factor(s) (Kato *et al*, 1992). Obviously, if DNA-bound factor E4 stabilizes DNA binding of factor E3 to an E3-binding half-site, it would potentially stabilize binding of E3 to the wild-type palindromic E3 binding site. Interestingly, PU.1 can bind to the κ 3' enhancer, but is unable to activate transcription, in the absence of NF-EM5 (Pongubala *et al*, 1993). An analogous effect with the putative E3 (c.f. NF-EM5) and E4 (c.f .PU.1) proteins binding to site FP-e correlates with my data, as discussed below.

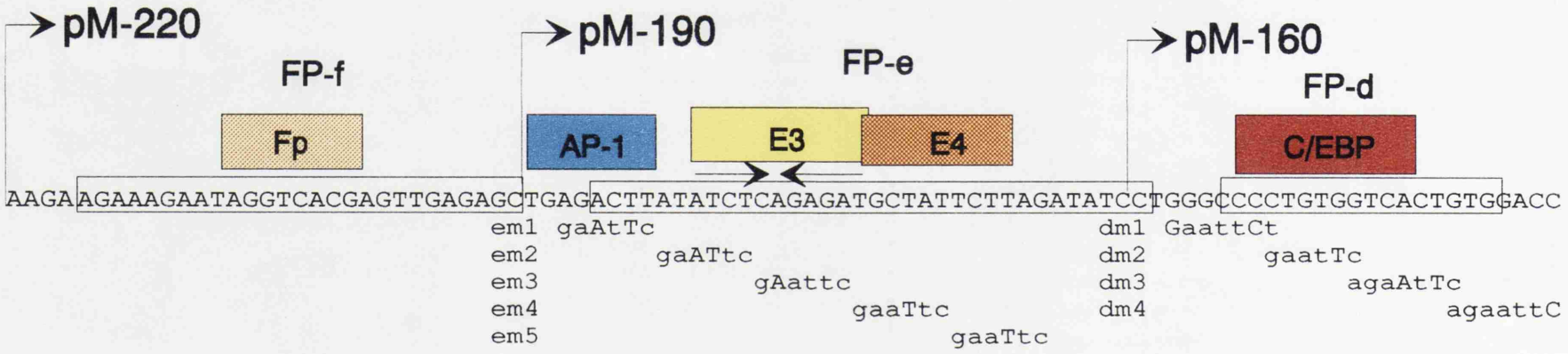
This model is consistent with the idea that C/EBP family proteins bound to site FP-d interact with a factor binding to FP-e, which in turn recruits further transcription factors which bind more distally. In order to complete the model, I will henceforth refer to the constitutively-acting positive-regulatory transcription factor(s) binding to the FP-f site as factor F_p. The proposed model is shown schematically in figure **16.5**.

In the following paragraphs, I will suggest how the model can account for the functional effects of mutations to site FP-e introduced into the pM-190 and pM-350 MIP1α promoters.

In unstimulated RAW 264.7 cells, factors E3 and E4 would bind to the FP-e site, largely occluding DNA binding by AP-1 (figure **16.5**), and therefore positively regulating transcription. Although the footprinting data suggests that AP-1 does not bind *in-vitro*, it would be expected to compete with factor E3 for DNA binding, as the proposed E3 DNA binding site is directly adjacent to the proposed AP-1 binding site (figures **16.5** and **16.6**). Mutation em1 in the pM-190 promoter should prevent DNA binding of AP-1, thus allowing unhindered DNA binding of factor E3, resulting in a small rise in promoter activity in unstimulated RAW 264.7 cells, as is observed (figure **16.6**). This supports the notion that AP-1 can act as a negative regulator of MIP1α promoter activity, and suggests that the AP-1 binding site might be a more potent negative regulatory site in TGFβ-treated RAW 264.7 cells. The fact that the pM-190em1 promoter has similar LPS-stimulated activity to the wild-type pM-190 promoter (figure **16.6**) suggests that the negative-regulatory effects of AP-1 were diminished during the period of LPS stimulation, consistent with the possible presence of different AP-1 complexes at this site during the LPS stimulation (see earlier discussion).

As discussed above, mutation em2 should reduce-, and mutation em3 abolish- DNA binding by positive-regulatory factor E3 (figure **16.6**), resulting in increased- and unrestricted- binding, respectively, of negative-regulatory AP-1 to the adjacent DNA site in the pM-190 promoter (figures **16.5** and **16.6**). This correlates with the observed reduction (in pM-190em2) and abolishment (in pM-190em3) of pM-190 promoter activity in unstimulated RAW 264.7 cells (figure **16.6**).

Upon LPS stimulation of RAW 264.7 cells, promoter pM-190em2 is induced 2.2-fold, similarly to the wild-type pM-190 promoter (figure **16.6**), and its reduced total LPS stimulated activity compared to pM-190 (figure **16.6**) correlates with the reduced DNA binding of factor E3. Assuming that factor E4 does not positively stimulate pM-190 promoter activity in the absence of E3, and that AP-1 negative-regulatory activity



	RAWCM	RAWCM+LPS
pM-350	38.5	33.8 (0.9)
pM-350dm1	7.3	11.3 (1.5)
pM-350dm2	12.7	20.6 (1.6)
pM-350dm3	7.9	21.1 (2.7)
pM-350dm4	45.1	41.8 (0.9)
pM-350em3	27.3	35.1 (1.3)
pM-350em4	1.4	0.9 (0.6)

	RAWCM	RAWCM+LPS
pM-160	5.0	19.7 (3.9)
pM-190	19.7	44.7 (2.3)
pM-190em1	29.5	44.2 (1.5)
pM-190em2	12.6	27.9 (2.2)
pM-190em3	0.6	15.2 (25.3)
pM-190em4	0.6	10.0 (16.7)
pM-190em5	2.0	16.6 (8.3)

Figure 16.6: Functional transient transfection analysis in RAW 264.7 cells of pM-190- and pM-350 MIP1a promoters containing point-mutations in the FP-d- and FP-e footprinted sites.

Top of figure: Coding-strand DNA sequence of the region of the MIP1α gene promoter containing footprinted sites FP-d, FP-e and FP-f. The sites corresponding to the 5' ends of the pM-160-, pM-190- and pM-220 MIP1α promoters are shown. The sequences corresponding to footprints FP-d, FP-e and FP-f in RAW 264.7 cells are contained within open boxes. Coloured boxes above the sequence span the proposed core binding sites for C/EBP family proteins (in FP-d), factors E4 and E3 (in FP-e), AP-1 (overlapping the 5' end of FP-e) and Fp (in FP-f), as shown. Point mutations dm1, dm2, dm3 and dm4 inserted into site FP-d of the pM-350 promoter, em3 and em4 inserted into site FP-e of the pM-350 and pM-190 promoters, and em1, em2 and em5 inserted into site FP-e of the pM-190 promoter, are denoted by lower-case letters below the relevant sites of the wild-type promoter sequence. Arrows above the FP-e sequence denote the inverted repeat sequence proposed to form the core binding site for putative transcription factor E3. **Bottom of figure:** Relative activities of mutant and wild-type MIP1α promoter/ hGH gene constructs transiently transfected into RAW 264.7 cells and incubated in RAW CM in the absence (RAW CM) or presence (RAW CM + LPS) of 2 μg/ml of LPS. hGH gene expression was expressed relative to that of the pHSVTKGH positive control, which was given a value of 1 in RAW 264.7 cells in RAW CM. Fold-induction of expression by RAW CM + LPS over the level of expression in RAW CM is shown in parentheses.

is reduced in response to LPS, promoter pM-190em3 should have similar-, though somewhat reduced- (due to the presence of residual negative-regulatory AP-1), total activity compared to promoter pM-160 after LPS stimulation, as is observed (figure **16.6**).

Mutation em4 in the pM-190 promoter should abolish E4 DNA binding (figure **16.6**), hence abolishing E3 DNA binding. Further, a second potential AP-1 DNA binding site in the FP-e site is created by mutation em4 (discussed above, and figure **16.6**). Thus, in unstimulated RAW 264.7 cells, the unrestricted DNA binding of AP-1 to two adjacent sites in the FP-e site would be expected to abolish promoter activity, as is observed (figure **16.6**).

In LPS-stimulated RAW 264.7 cells, the DNA binding of AP-1 to the FP-e site would be reduced, but due to the presence of two adjacent sites, compared to only one in pM-190em3, greater AP-1-mediated negative regulation of pM-190em4 than pM-190em3 in LPS-stimulated RAW 264.7 cells might be expected, so that the total LPS-stimulated activity of pM-190em4 would be somewhat lower than that of pM-190em3; this is what is observed (figure **16.6**).

Mutation em5 in the pM-190 promoter significantly reduces the activity of the promoter in unstimulated RAW 264.7 cells, but the activity is still 3-fold greater than that of pM-190em3 and pM-190em4 (figure **16.6**). The simplest explanation for this is that mutation em5 overlaps the DNA binding site of factor E4 (figure **16.6**), significantly reducing but not abolishing DNA binding of E4, and hence of E3, and therbeby significantly reducing but not abolishing pM-190 promoter activity, as is observed (figure **16.6**). As mutation em5, in contrast to em4, does not introduce a second potential AP-1 DNA binding site into the FP-e site (figure **16.6**), the LPS-induced activity of pM-190em5 would be expected to be greater than that of pM-190em4, as is observed (figure **16.6**).

It is possible that protein-protein interaction between factors E3 and E4 binding to the FP-e sequence and factor F_p to the FP-f sequence is sufficient to occlude AP-1 DNA binding at the overlapping site (figure **16.5**), as is observed in footprinting experiments. Mutation em3 in the pM-190 promoter (figure **16.6**) was proposed to

abolish recruitment of factor E3 to its DNA binding site by factor E4. However, it is feasible that factor E3 interacts with both factors E4 and F_p, and that this complex could bind to DNA even in the presence of the em3-mutated E3 DNA binding site, though with reduced affinity compared to binding to the wild-type template. Hence, in unstimulated RAW 264.7 cells, the lower affinity of the E4-E3-F_p complex for the DNA would allow greater competition for DNA binding by AP-1, slightly reducing the activity of the pM-350em3 promoter compared to wild-type pM-350, and this is what is observed (figure figure **16.6**). In LPS-stimulated RAW 264.7 cells, the reduced negative-regulatory effects of AP-1 would allow the E4-E3-F_p complex to regulate the activity of pM-350em3 similarly to that of wild-type pM-350, as is observed (figure **16.6**).

Mutation em4 in promoter pM-350 should abolish DNA binding of factor E4 (figure **16.6**), and hence of factor E3, to the FP-e site, thus possibly allowing DNA binding of AP-1 to two adjacent sites in the mutated FP-e sequence (figure **16.6**). As proposed above, AP-1 appears to compete with factor E3 for DNA binding to site FP-e in the wild-type promoter (figures **16.5** and **16.6**). In the same way that E3 is presumed to interact with the adjacent E4 and F_p factors, it is possible that AP-1 DNA binding to this site might be stabilized by transcription factors bound to the adjacent DNA. In the mutant pM-190em4 promoter, the FP-f site is not present and the E4 site is mutated (figure **16.6**), but in the mutant pM-350em4 promoter, the FP-f site is present. Hence, it is possible that in the context of the pM-350em4 promoter, factor F_p and the two adjacently-bound AP-1 factors stabilize each others' binding to the promoter, resulting in a negative-regulatory complex. In LPS-stimulated RAW 264.7 cells, though DNA binding of the two AP-1 complexes alone might be expected to only reduce the LPS-stimulated pM-350em4 promoter activity (as for the pM-190em4 promoter), the increased stability of the F_p-AP-1-AP-1 negative-regulatory complex might prevent promoter activation by LPS, as is observed (figure **16.6**).

As I have proposed that binding of transcription factors (other than AP-1) to the distal region of the MIP1α promoter may be dependent on C/EBP family protein

binding to the proximal promoter, this suggests that the proposed inhibition by LPS-induced CHOP protein of C/EBP family proteins binding to the proximal promoter (see earlier discussion) may also result in a loss of transcription factor binding to the distal promoter, and this possibility is depicted in the model shown in figure **16.5.**

Although I do not expect the above model to be entirely accurate, the proposed co-operative binding of transcription factors to the MIP1α promoter could easily be tested in EMSAs, using PCR-generated probes encompassing different lengths of the described regions. Further, as the discussed functional data suggests interactions between factor(s) binding to the FP-f region of the promoter and the FP-e and FP-d sites, functional testing of pM-350 promoters containing point mutations of this region would be informative.

16.10 REGULATION OF MIP1α GENE EXPRESSION BY INTERFERONS.

16.10.1 Negative regulation of the MIP1a promoter in RAW 264.7 cells by IFNγ.

IFNγ is reported to up-regulate MIP1α mRNA levels 1.5-fold in J774.2 macrophages (Martin *et al*, 1991). As an induction of 1.5-fold is rather small, this could perhaps be accounted for by the presence of slightly different levels of fresh serum in control- compared to IFNγ-stimulated cells, which would be in agreement with my work, in which I found that IFNγ did not induce MIP1α mRNA in RAW 264.7 cells, and in fact antagonised the LPS- or fresh serum-induced upregulation of MIP1α mRNA levels in these cells.

Although I have not investigated the effects of IFNγ on MIP1α gene transcription in RAW 264.7 cells, IFNγ induces transcription of the IP-10 gene (a PF4 family member) in RAW 264.7 cells (Ohmori *et al*, 1993). Maximal responsiveness of IP-10 gene promoter constructs to IFNγ requires an ISRE element plus two κB elements, one of which is homologous to the MIP1α promoter κB element (Ohmori *et al*, 1993), though an IFNγ response is still observed when the other, canonical, κB element is mutated. Hence, the MIP1α promoter, which contains an ISRE (in FP-a,

figure **16.7**) and a κB element (in FP-b, figure **16.4**), is potentially responsive to IFNγ treatment of RAW 264.7 cells.

As both LPS and IFNγ activate macrophages, and co-treatment of macrophages with these agents can enhance macrophage activation (Chen *et al*, 1989), it may appear counter-intuitive that MIP1α gene expression should be up-regulated by LPS and that IFNγ should antagonize this induction, especially in light of the IP-10 data discussed above. Interestingly CRG-10, another PF-4 family gene, is induced by IFNγ-, but not LPS-, treatment of RAW 264.7 cells (Farber, 1992). Moreover, like the MIP1α gene in RAW 264.7 cells, expression of the *c-fos* early-response gene is up-regulated (at the level of transcriptional elongation) by LPS- but down-regulated by IFNγ- (at the level of mRNA stability) treatment- of macrophages (Collart *et al*, 1991; Radzioch *et al*, 1991), suggesting MIP1α mRNA may be destabilized by IFNγ treatment of RAW 264.7 cells.

Though the ISRE of the IP-10 gene is functionally IFNγ-responsive in RAW 264.7 cells, the MIP1α promoter ISRE overlaps several other transcription factor binding sites, for example those for PU.1/Spi-B and C/EBP family proteins (figure **16.7**). Hence, as IFNγ stimulation of RAW 264.7 cells leads to only low levels of ISRE-specific complex formation in EMSAs (Ohmori *et al*, 1993), this may not be sufficient to compete with PU.1/Spi-B or C/EBP family proteins for binding to the MIP1α promoter FP-a site, so that a transcriptional response of the MIP1α gene to IFNγ stimulation of RAW 264.7 cells may not be observed.

A further interesting possibility, which may occur in addition to the destabilization of MIP1α mRNA by IFNγ, stems from the fact that, like LPS, IFNγ is cytostatic for macrophages (Vairo *et al*, 1992), so that IFNγ stimulation may induce CHOP expression in RAW 264.7 cells (Fornace Jr *et al*, 1989; Ron *et al*, 1992), hence preventing C/EBP family proteins from binding to the MIP1α proximal promoter and thereby down-regulating MIP1α transcription. In this scenario, unlike LPS, IFNγ would not induce positive regulatory C/EBP protein levels, so that no IFNγ-stimulated transient induction of MIP1α transcription via C/EBP proteins would occur. In testing this theory, it is likely that, similarly to the LPS-stimulated

Figure 16.7: Sequences homologous to MIP1α promoter site FP-a.

Sequence alignment of the MIP1α promoter site FP-a with FP-b (figure **11.1**) and documented transcription factor binding sites ISRE (Fu, 1991), E-box (Metz *et al*, 1991), C/EBP (Landchultz *et al*, 1988) and PU.1 (Klemsz *et al*, 1990), and the CK-1 cytokine promoter motif (Nishizawa *et al*, 1990; Shannon *et al*, 1990). (c) and (nc), coding and non-coding DNA strands, respectively.

```
FP-a (c)   CCCAGATTCCATTTCCTCATCTGCTAGG
ISRE         CAGtTTCnnTTTCC
CK-1         gAGATTCCAc
E-box                        CAnnTG
FP-b               aatTTTCC

FP-a (nc)  CCTAGCAGATGAGGAAATGGAATCTTGG
C/EBP            atTGcGcAAt
PU.1             aAaGAGGAAc
```

induction of CHOP proposed earlier, high levels of IFNγ would be required to arrest the proliferation of the RAW 264.7 cells and hence observe any CHOP-mediated effects.

An interesting extension to this theory is that, in stimulating the removal of C/EBP family proteins from the MIP1α promoter, IFNγ may thereby allow access to the promoter of other transcription factors whose DNA binding sites were previously occluded by the binding of C/EBP family proteins to overlapping sites.

16.10.2 Is the MIP1α promoter ISRE functional in RAW 264.7 cells ?

Preliminary experiments suggest that IFNα/β treatment up-regulates MIP1α mRNA levels in RAW 264.7 cells, and that pretreatment of these cells with IFNγ enhanced this effect. Further, as the MIP1α promoter FP-a site contains an ISRE, this suggests that the response may at least in part be at the level of transcription.

Of the IRF family of transcription factors, only the ISGF3γ protein interacts with the IFNα/β-inducible ISGF3α component of the ISGF3 transcription factor, that positively regulates transcription of IFNα/β-responsive genes via the ISRE element (Schindler *et al*, 1992; Fu, 1992; Veals *et al*, 1992). IFNγ pretreatment of multiple cell types, including the human monocytic cell line U937, up-regulates the levels of ISGF3γ, so that more of this protein is available for interaction with IFNα/β-induced ISGF3α, thus increasing the magnitude of the transcriptional response to IFNα/β (Schindler *et al*, 1992; Fu, 1992; Improta *et al*, 1992). Although the induction by IFNγ of ISGF3γ levels in mature macrophages has not been investigated, pretreatment of RAW 264.7 cells with IFNγ appeared to enhance the induction of MIP1α mRNA by IFNα/β, suggesting that ISGF3γ levels in RAW 264.7 cells may be induced by this pretreatment. A further possibility, in keeping with the above discussion, is that IFNγ pretreatment of RAW 264.7 cells also increases the accessibility of the MIP1α promoter ISRE to ISGF3.

As discussed earlier, LPS treatment of RAW 264.7 cells stimulates IFNα/β secretion, which feeds back onto the cells and induces transcription via ISGF3 binding to ISRE promoter elements. This raises the question of why no ISRE-specific transcription factor complex (presumably ISGF3) is observed to bind in EMSAs to

MIP1α promoter FP-a probe using nuclear extracts from LPS-stimulated RAW 264.7 cells ? Examination of EMSAs using FP-a probe and nuclear extracts from RAW 264.7 cells stimulated with LPS over a timecourse (figure **11.9**) does reveal a complex migrating more slowly than complex 1/a1 after 6 hours of LPS stimulation, correlating with the known slow migration in EMSAs of the ISGF3 complex (Schindler *et al*, 1992; Fu, 1992). However, the relatedness of this FP-a probe complex to ISGF3 or to C/EBP family proteins has not been examined.

Two reasons for why an ISGF3 complex should not be formed with the FP-a probe are: 1) the FP-a "ISRE" has 1 bp change from a consensus ISRE (figure **16.7**), and the effects of this difference on the ability of the ISRE to bind ISGF3 have not been examined; 2) ISGF3 is a tyrosine-phosphorylated protein complex and its DNA binding is abolished by treatment with a specific tyrosine-phosphatase (Fu, 1992); this can be prevented by incorporation of 1 mM sodium orthovanadate into the incubation of the complex with the phosphatase (Fu, 1992). As the preparation of 1 mM sodium orthovanadate is time-consuming, once its presence was shown not to be required (for example in the study of C/EBP family proteins), it was not included in buffers used to prepare and analyse proteins present in nuclear extracts. In such nuclear extracts, the binding of ISGF3 to the MIP1α ISRE would probably not be observed.

16.11 THE FP-a SITE OF THE PROXIMAL MIP1α PROMOTER.

I have proposed that in unstimulated RAW 264.7 cells, the FP-a site is predominantly bound by either PU.1 or Spi-B, whereas in LPS-stimulated RAW 264.7 cells it is predominantly bound by C/EBPβ1/2/δ (see figure **16.5**). In addition, c-Rel and/or a c-Rel-containing complex present in RAW 264.7 cell nuclear extracts can bind to FP-a. Furthermore, FP-a also contains an ISRE, and a CK-1 motif, and all of these proposed or identified transcription factor binding sites are either overlapping or juxtaposed (figure **16.7**).

Generally when two transcription factor DNA binding sites overlap, only one of those factors can bind to the DNA. However, it has been demonstrated that, though

the tight juxtaposition of C/EBP- and YY-1 binding sites on the serum albumuin promoter allows C/EBP to impair the binding of YY-1 to this site *in-vitro*, the two factors synergistically stimulate transcription, as YY-1 allows C/EBP to induce formation of preeinitiation complexes that are stable through multiple rounds of transcription (Milos *et al*, 1992). It is therefore possible, for example, that the tight juxtaposition of the CK-1 site to the C/EBP family- and PU.1/Spi-B binding sites in the MIP1α promoter FP-a sequence may allow DNA binding of a transcription factor(s) to the CK-1 sequence in the presence of one or other of these adjacently-bound factors. In support of this is the fact that both the CK-1 motif and C/EBP family binding site in the murine G-CSF gene promoter mGPE-1 sequence are directly adjacent, and both are required for LPS-inducibility of this promoter in a macrophage cell line, although in EMSAs the only observed binding to mGPE-1 was that by C/EBP family members (my data; Nishizawa *et al*, 1990).

For the other factors implicated or observed to be able to bind to the FP-a sequence, to do so *in-vivo* in macrophages they would be required to displace PU.1/Spi-B or C/EBP family members. This may suggest that c-Rel and/or interferon-induced proteins would be more important in the regulation of transcription of the MIP1α gene in non-macrophage cell types. For example, although expressed at only a low level in myeloid cells, c-Rel is expressed at very high levels in lymphoid cell types (Nakayama *et al*, 1992). Similarly, most cell types express cell-surface receptors for IFNγ and IFNα/β (Merlin *et al*, 1985), raising the possibility that these agents may induce the MIP1α gene via the FP-a site ISRE in non-macrophage cell-types. Hence, the FP-a site of the MIP1α promoter may be similar to the SRE region of the *c-fos* early-response gene promoter, which contains overlapping binding sites for multiple transcription factors, which bind to the SRE in a cell type-specific and cell stimulus-dependent manner (see chapter **3**), thus allowing transcriptional-responsiveness of the *c-fos* promoter to multiple signals in a cell type-specific manner. Interestingly, the *c-fos* SRE can bind C/EBPβ and the helix-loop-helix protein E12 at overlapping sites, and the FP-a sequence contains the sequence CATCTG, overlapping the C/EBP family binding site, and this sequence is identical

to the core of the E12 protein binding site in the *c-fos* SRE (Metz *et al*, 1991; figure **16.7**). However, in addition to the CAnnTG (the "E-box") sequence, flanking sequences are important in determining DNA binding of HLH proteins, and E12 does not bind the CTCATCTG sequence present in FP-a (Metz *et al*, 1991). Nevertheless, as the E-box is the core binding site for multiple bHLH and hHLH-LZ proteins (see chapter **2**), this suggests that further HLH proteins could regulate transcription of MIP1α via the FP-a site in a cell-specific manner.

CHAPTER 17: PERSPECTIVES- WHAT THIS THESIS SUGGESTS ABOUT THE POSSIBLE ROLES OF MIP1α PROTEIN *IN-VIVO*.

17.1 MIP1α AND THE INFLAMMATORY RESPONSE.

The modulation of MIP1α gene expression in RAW 264.7 cells by LPS, 10% (v/v) FCS, IFNγ, IFNα/β and TGFβ is consistent with a role for macrophage-produced MIP1α in the processes of wound-healing and inflammation, as growth factors and cytokines, including the above, are produced at sites of injury or infection (Rappolee *et al*, 1989). As MIP1α is chemotactic for monocytes, which are recruited to inflammatory sites (Schall, 1991; Rappolee *et al*, 1989), modulation of the production of MIP1α at such sites may be affect this recruitment of monocytes.

17.2 REGULATION OF MIP1α TRANSCRIPTION IN TISSUE-MACROPHAGES: ARE RAW 264.7 CELLS A GOOD MODEL ?

A feature of RAW 264.7 cells and other cultured macrophage cell lines is that they are continuously proliferating, while resident tissue macrophages are differentiated and not proliferating (Gordon *et al*, 1986); this suggests that the non-DNA binding negative-regulatory C/EBP family member CHOP (Fornace Jr. *et al*, 1989; Ron *et al*, 1992) may be expressed in tissue macrophages. In support of this, when 3T3-L1 cells, which do not contain CHOP protein, are induced to differentiate into adipocytes, CHOP expression is induced (Ron *et al*, 1992). Hence, the possibility

exists that C/EBPβ and -δ are more important in the regulation of the MIP1α gene in macrophages in inflammatory situations, and that high levels of CHOP in tissue macrophages may act to "mop-up" excessive negative-regulatory C/EBPβ3 or positive-regulatory C/EBPβ1/2/δ, so contributing to the maintenance of a low level of constitutive production of MIP1α protein by macrophages in the bone marrow.

A further intriguing possibility is that, although C/EBP family- and Rel family proteins may regulate MIP1α transcription in RAW 264.7 cells, the presence of CHOP in resident tissue macrophages may allow the reprogramming of MIP1α transcription in these cells, such that the gene may be regulated via some of the regulatory elements that are obscured by the binding of C/EBP family proteins in RAW 264.7 cells. Thus, the proposed presence of CHOP in tissue-macrophages and absence in macrophage cell-lines suggests that RAW 264.7 cells may be a good model for inflammatory macrophages but not for tissue-macrophages involved in the regulation of stem cell proliferation.

17.3 MIP1α AND CANCER.

The involvement of several proto-oncogenic transcription factor families, ie. ETS, Rel, Fos and Jun, in the transcripional regulation of the MIP1α gene suggests a potential for disregulation of MIP1α gene expression. Furthermore, as I have suggested that CHOP may play a role in maintaining the correct levels of MIP1α gene expression in tissue macrophages, the observation that a chromosomal translocation event that results in production of an abnormal CHOP fusion protein is characteristic of certain human tumour cells (Crozat *et al*, 1993) suggests the potential involvement of CHOP in disregulated MIP1α gene expression. If the presence of MIP1α in the bone marrow is important in the regulation of stem cell proliferation, excessive production of MIP1α in bone marrow macrophages, due to the presence of an oncogenic version of one of the above transcription factors or an abnormal form of CHOP, would be expected to have important consequences. This correlates with the observation of elevated levels of MIP1α mRNA in the peripheral

blood and/or bone marrow of patients with certain leukaemic- and pre-leukaemic disorders (Maciejewski *et al*, 1992; Yamamura *et al*, 1989).

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