Biological and Genetic Characterisation of a Novel Murine Erythroleukaemia System.

by

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For Mum, Dad, Richard and Gilly.

Abstract.

The ELM erythroleukaemia is novel in that *long-term* survival of leukaemic cells in culture (ELM-D cells) is dependent on contact with a bone marrow-derived stromal feeder cell layer. A collaborative study has shown that the transmembrane form of stem cell factor is a crucial component of the stromal cell-derived survival signal. A number of stroma-independent (ELM-I) mutants have been derived that vary in their tumorigenicity and their ability to differentiate *in vitro* in response to erythropoietin and IL-3. The work in this thesis has attempted to define the genetic changes responsible for these different phenotypes. At the *p53* locus in the primary leukaemic cells, one copy of the gene has been lost whilst the other contains an 18bp deletion, implicating its mutation as an early step in the development of the leukaemia. Changes in *ets* gene expression have also been found. The *Fli-1* locus is rearranged in the primary tumour due to the insertion of a retrovirus upstream of one *Fli-1* allele. However, this does not result in *Fli-1* gene activation in any of the ELM-D or ELM-I cell lines, except one, which significantly is the only cell line to have lost the ability to differentiate in response to erythropoietin. In addition, up-regulation of another *ets* family member, *erg*, is associated with stromal cell-independent growth since all ELM-I mutants have moderate levels of *erg* mRNA, whereas only low or undetectable levels are found in primary leukaemic cells *in vivo* or in ELM-D cells *in vitro*. This up-regulation of *erg* mRNA seems to be important for stromal cell-independent growth since ELM-D cells, which exhibit *short-term* viability after separation from stromal cells, show elevated expression of the *erg* gene. This seems to be made permanent in ELM-I mutants since, unlike ELM-D cells, they do not down-regulate *erg* mRNA when grown in contact with stromal cells. These experiments suggest that alterations in *ets* gene expression may affect both the survival and the differentiation of erythroid cells. Future work designed to assess the validity of this hypothesis is discussed.

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Abbreviations.

A	: Adenine.
α-MEM	: Alpha minimal essential medium.
AEV	: Avian erythroblastosis virus.
A_x	: Absorbance $_{\text{wavelength (nm)}}$.
b	: Base.
BFU-E	: Burst-forming unit-erythroid.
bp	: Base pair.
BPA	: Burst-promoting activity.
C	: Cytosine.
°C	: Degrees centigrade.
c-	: Cellular.
cDNA	: Complementary deoxyribonucleic acid.
CFU-E	: Colony-forming unit-erythroid.
$CHCl_3$	: Chloroform.
Ci	: Curie.
CSF-1	: Colony-stimulating factor-1.
DEPC	: Diethylpyrocarbonate.
DMSO	: Dimethylsulphoxide.
DNA	: Deoxyribonucleic acid.
DNAse	: Deoxyribonuclease.
dNTP	: 3' deoxyribonucleoside 5' triphosphate.
DTT	: Dithiothreitol.
ECM	: Extracellular matrix.
EDF	: Erythroid differentiation factor.
EDTA	: Ethylenediaminetetra-acetic acid, disodium salt.
EGF	: Epidermal growth factor.
ELM-D	: Stromal cell-dependent ELM erythroleukaemia cells.
ELM-I	: Stromal cell-independent ELM erythroleukaemia cells.
env	: Envelope.
EPA	: Erythroid-potentiating activity.
epo	: Erythropoietin.
epoR	: Erythropoietin receptor.
ESI	: Elk-1 SRF interaction domain.
F-MuLV	: Friend murine leukaemia virus.
Fn	: Fibronectin.
ϕOH	: Phenol.
FV	: Friend virus.
FV-A	: Friend virus (anaemia-inducing strain).
FV-P	: Friend virus (polycythaemia-inducing strain).
g	: Gram.
G	: Guanine.
GAPDH	: Glyceraldehyde phosphate dehydrogenase.
gag	: Group antigen.
GM-CSF	: Granulocyte-macrophage colony-stimulating factor.
gp	: Glycoprotein.
Hepes	: N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid).

HLH : Helix-loop-helix.
HPV : Human papillomavirus.
hr : Hour.
IL-1 : Interleukin-1.
IL-2 : Interleukin-2.
IL-3 : Interleukin-3.
IL-7 : Interleukin-7.
k : Kilo.
l : Litre.
LFS : Li-Fraumeni syndrome.
LTBMC : Long-term bone marrow culture.
LTR : Long terminal repeat.
μ : Micro.
m : Milli.
M : Molar.
mA : Milliamps.
MCF : Mink cell focus-forming virus.
min : Minute.
mm : Millimetre.
MOPS : 3-(N-morpholino) propanesulphonic acid.
mRNA : messenger ribonucleic acid.
n : Nano.
NaAc : Sodium acetate.
p : Pico.
'phage : Bacteriophage.
PBS : Phosphate-buffered saline.
PCR : Polymerase chain reaction.
pfu : Plaque-forming unit.
PIPES : Piperazine-N, N'-bis (2-ethanesulphonic acid).
pol : Retroviral polymerase.
RA : Retinoic acid.
Rb : Retinoblastoma.
REF : Rat embryo fibroblast.
RNA : Ribonucleic acid.
RNAse : Ribonuclease.
RNAsin : Ribonuclease inhibitor.
rNTP : 3' ribonucleoside 5' triphosphate.
rpm : Revolutions per minute.
rRNA : Ribosomal ribonucleic acid.
RT : Reverse transcriptase.
RTemp : Room temperature.
sec : Second.
SCF : Stem cell factor.
SDS : Sodium dodecyl sulphate.
SFFV : Spleen focus-forming virus.
Sl : *Steel* locus.
Sl^d : *Steel-Dickie* locus.
SRE : Serum response element.
SRF : Serum response factor.

SV40	: Simian virus 40.
T	: Thymine.
T3	: Thyroid hormone.
TEMED	: Tetramethylenediamine.
TGF-α	: Transforming growth factor-alpha.
TM	: Transmembrane.
TNF-α	: Tumour necrosis factor-alpha.
Tris	: 2-amino-2-(hydroxymethyl) propane-1,3-diol.
tRNA	: Transfer ribonucleic acid.
ts	: Temperature sensitive.
U	: Units.
UV	: Ultra violet.
V	: Volts.
v-	: Viral.
v/v	: Volume for volume.
W	: *White-spotting* locus.
w/v	: Weight for volume.
wt	: Wild-type.
X-gal	: 5-bromo-4-chloro-3-indoyl-β-D-galactoside.

Amino acid one letter and three letter code used in this thesis is the same as that described by Sambrook *et al*, 1989.

Throughout this thesis genes/alleles are indicated by italics e.g. *Fli-1*, whilst proteins remain unitalicised e.g. Fli-1.

PART 1: INTRODUCTION.

CHAPTER 1: The Control of Erythropoiesis.

1.1: Haemopoiesis and leukaemia.

During normal steady-state haemopoiesis in the mouse, blood cells are continually being renewed, as old cells are removed by the liver and replaced by a tightly controlled system of proliferation and differentiation of cells within the bone marrow and the spleen (Figure 1.1) (for a review, see Dexter and Spooncer, 1987). It has been estimated that in humans $3.7x10^{11}$ blood cells need to be produced per day to replace those lost by natural wastage: injury, infection and haematological stress increases and modifies this demand for cell production. All these mature cells are derived from a small number of pluripotent stem cells in the haemopoietic organs. These cells are able to undergo indefinite self-renewal, but also generate cells that are more restricted in terms of their developmental potential. These are essentially of two types: the lymphoid precursor cell is only able to produce mature lymphocytes, of both the T and B lineages, whilst the multipotential myeloid precursor cell can generate all the remaining blood cells i.e. mast cells, erythrocytes, neutrophils, macrophages, eosinophils and megakaryocytes (that form platelets). Throughout this thesis, 'myeloid' is defined as all the non-lymphoid cells and their precursors in recognition of the existence of a common precursor cell, including erythroid cells but excluding pluripotent stem cells.

The precursors produce daughter cells that become committed to one particular lineage. It is at this stage of differentiation when cell numbers are rapidly expanded, such that the number of mature cells formed per precursor is immense. These cells respond to maturation signals that activate lineage-specific genes which give the mature cell its characteristic properties. This whole process is responsive to a large number of cytokines and regulatory molecules that stimulate or repress the proliferation, survival and maturation of specific cell types. In steady-state haemopoiesis, it appears that a significant proportion of this control is exerted locally by the production of these regulators by stromal and haemopoietic cells in the

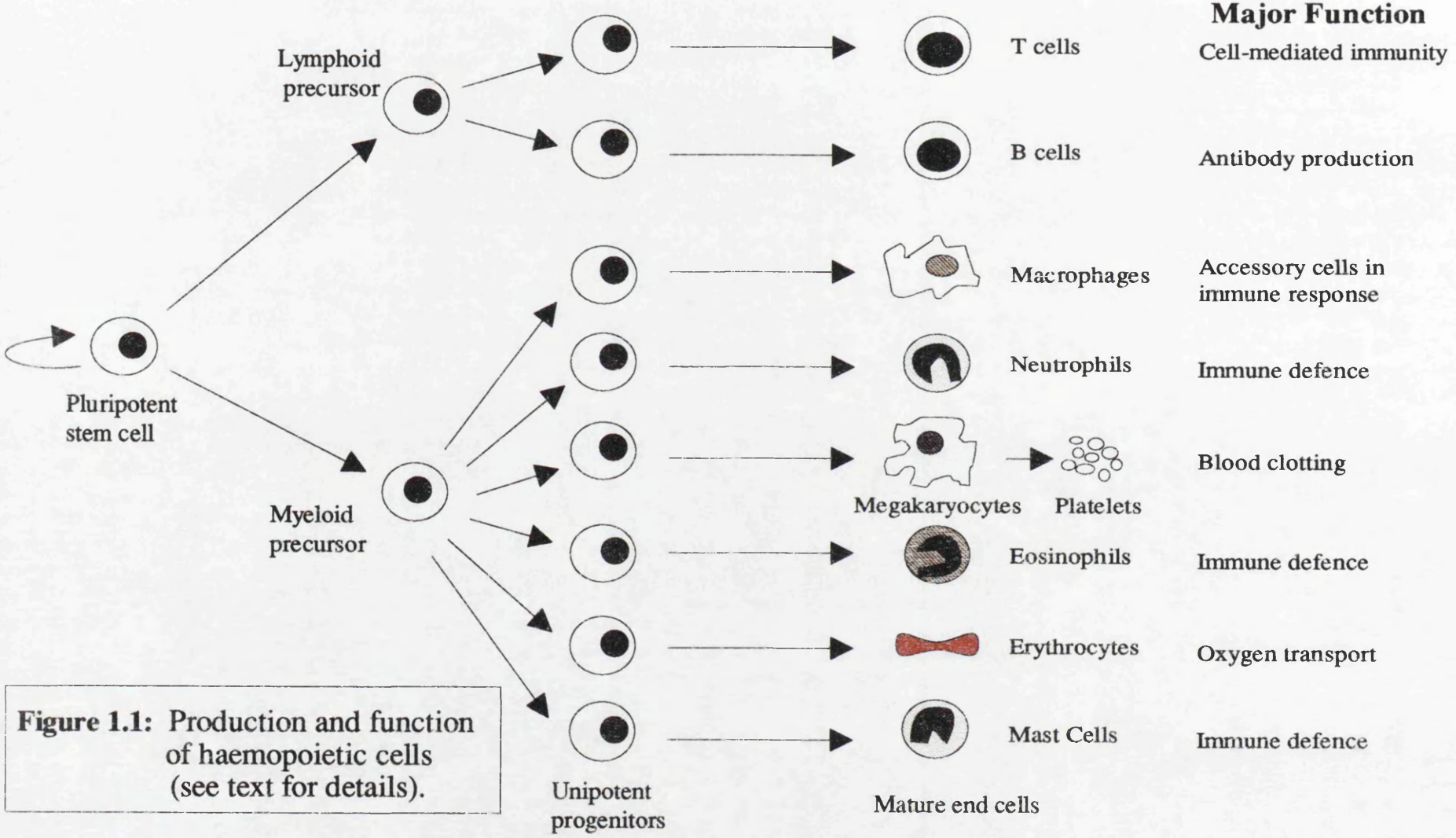


Figure 1.1: Production and function of haemopoietic cells (see text for details).

haemopoietic organs. Thus, to a certain extent, these organs act autonomously. However, a crucial facet of the system is that it must be able to respond to the needs of the animal in times of stress, for example during chronic infection or injury-induced blood loss. The precursor and progenitor cell populations are able to respond to cytokines produced as a consequence of these events, such that the number and type of blood cell produced meets these requirements. Furthermore, it also seems likely that stress-induced regulators also alter the spectrum of cytokines produced in the haemopoietic microenvironment. These two distinct control mechanisms has meant that the role of different regulatory molecules, either in 'emergency' or steady-state haemopoiesis, is often a matter of some controversy; an *in vitro* response of progenitors cells to a particular cytokine does not define the exact role of that molecule *in vivo*, but simply indicates the cell's ability to respond. The question of when and why the cell needs or utilises this property remains, in many cases, unanswered.

In leukaemia, the normal control of haemopoiesis is disrupted. Progenitor, precursor or stem cells proliferate uncontrollably and become insensitive to maturation signals due to the accumulation of mutational events that alter the function of particular oncogenes and tumour suppressor genes (reviewed in Sawyers *et al*, 1991). Erythroleukaemias, the subject of this thesis, are relatively unusual in humans, representing less than 5% of leukaemias (Villeval *et al*, 1986). However, their study in animal model systems has given important insights into the multistage nature of cancer and the function of several genes that are also involved in human tumorigenesis.

This introductory section will concentrate on describing the molecules that may be involved in regulating normal murine erythropoiesis, and then detail erythroleukaemia systems that have been used to identify genes capable of abrogating this control. The current hypotheses concerning the role of these genes in normal cells will then be described in an attempt to understand why they are targeted during the development of erythroleukaemias. I will then describe the ELM cell system

which has provided a unique opportunity to examine the role of stromal cells in erythropoiesis and to identify the effect particular genes have on tumour progression within this lineage.

1.2: Erythropoiesis.

Distinct committed erythroid progenitors have been identified called the colony-forming unit-erythroid (CFU-E), a mature progenitor, and the burst-forming unit-erythroid (BFU-E), a more primitive cell (Figure 1.2). Within these two groups are cells at different stages of maturation, thus representing a continuum of development from the earliest unipotent progenitor cell to the mature erythrocyte. The earliest BFU-E cell is a lineage-restricted product of a myeloid precursor, a cell that has the potential to generate all other myeloid cells and is itself derived from a pluripotent stem cell. The existence of CFU-E cells was implied by the observation that small colonies of between eight and sixty haemoglobinised cells form when murine haemopoietic cells are cultured in semi-solid media solely in the presence of low levels of purified erythropoietin (epo) for four days (Stephenson *et al*, 1974). Each colony is believed to be derived from a single, rapidly-dividing CFU-E progenitor. In similar clonogenic assays, larger grouped colonies called 'bursts' appear after five to seven days, although the amount of epo incorporated in the assay has to be considerably higher (Gregory and Eaves, 1978). These bursts are derived from single late BFU-E cells, with earlier BFU-E forming larger bursts, taking longer to do so and requiring additional growth factors. BFU-E cells divide less frequently than CFU-E progenitors, but have the potential to generate more erythrocytes. The size of the CFU-E population is closely dependent on the serum epo levels. The survival, proliferation and maturation of the BFU-E cells, on the other hand, appears to be much less responsive to epo, but instead is probably responsive to a large number of molecules produced locally in the haemopoietic compartments to create an inductive microenvironment. Components of this are discussed below.

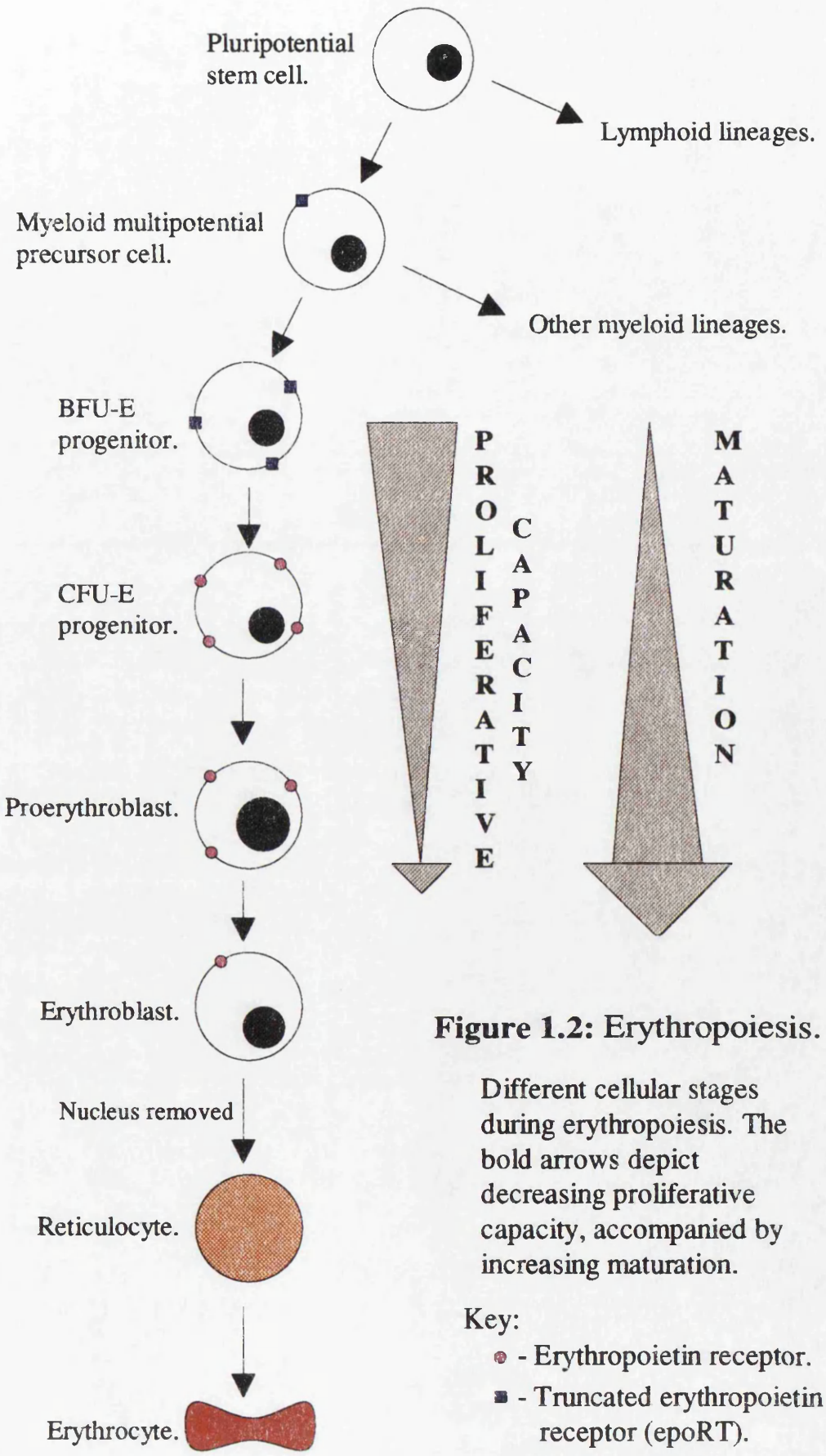


Figure 1.2: Erythropoiesis.

Different cellular stages during erythropoiesis. The bold arrows depict decreasing proliferative capacity, accompanied by increasing maturation.

Key:

- ● - Erythropoietin receptor.
- ■ - Truncated erythropoietin receptor (epoRT).

(see text for details).

The purification of these erythroid progenitor cells (Sawada *et al*, 1990; Koury *et al*, 1984; Nijhof *et al*, 1987) and the molecular cloning of the erythropoietin gene, and subsequent production of the recombinant protein (Jacobs *et al*, 1985; Lin *et al*, 1985), have been very important in understanding the later stages of erythropoiesis. CFU-E cells, in response to epo, develop through several morphologically distinct intermediate cell types, namely proerythroblast, erythroblast and reticulocyte, before the mature erythrocyte is formed. Gradually during maturation erythroid-specific genes are switched on that produce the proteins required in a functional erythrocyte e.g. α- and β-globins (reviewed in Krantz, 1991). Expression of these genes is dependent at least in part on the binding of the GATA-1 zinc-finger transcription factor to recognition sequences in their promoters; mature erythrocytes are not formed from cells lacking this protein (Pevny *et al*, 1991). However, the processes that determine whether a cell becomes committed towards erythrocyte production, and how erythroid-specific gene expression is coordinately regulated, remain largely unresolved. Finally, after this lengthy process of expansion and differentiation, the nucleus is removed and the mature red blood cell passes into the blood stream to perform its critical function as an oxygen transporter.

1.3: Regulation of erythropoiesis: Growth factors and the microenvironment.

1.3.1: Erythropoietin.

Erythropoietin (epo), the principal factor regulating changes in erythropoiesis in the mouse, is a small glycoprotein released into the blood by the kidney, and to a lesser extent by the liver (reviewed in Krantz, 1991). By a combination of transcriptional activation through an enhancer 3' to the *epo* gene (Pugh *et al*, 1991), and stabilisation of the *epo* mRNA (Goldberg *et al*, 1991), tissue hypoxia increases epo production. Epo binds to the epo receptor (epoR) expressed on erythroid progenitors (D'Andrea *et al*, 1989a), which is a member of the large 'class I' cytokine receptor family that lack intrinsic kinase activity and probably transduce signals by interacting with other membrane-associated proteins (reviewed in Bazan, 1990). In

particular, epoR is structurally-related to the interleukin-2 receptor β-chain, which forms part of an oligomeric complex that permits high affinity binding of the ligand and transduces a signal (Saragovi and Malek, 1990). The putative complex formed by ligand-associated epoR proteins generates second messengers in responsive CFU-E cells that prevent apoptosis (Koury and Bondurant, 1990), the process of programmed cell death characterised by membrane blebbing, chromatin condensation, DNA fragmentation and eventual cellular disintegration (reviewed in Bursch *et al*, 1992). As the CFU-E mature into erythroblasts their survival no longer requires epo, and epo receptors are lost from the cell surface (Figure 1.2) (Landschulz *et al*, 1989). Thus, epo allows more progenitor cells to mature through this stage of differentiation.

However, this is probably an over-simplistic view of the role of epo in haemopoiesis. It is likely that epo also stimulates less mature progenitors; erythroid 'bursts' can form in clonogenic assays simply on the addition of purified epo to the media (Gregory and Eaves, 1978), whilst the number of cycling human BFU-E cells *in vivo* is increased during epo infusion (Dessypris and Krantz, 1984). Interestingly, a truncated form of the epo receptor gene (*epoRT*) that lacks part of the cytoplasmic domain is preferentially expressed on earlier progenitors, stimulation of which is unable to block apoptosis but can still transduce a mitogenic signal (Figure 1.2) (Nakamura *et al*, 1992). Loss of this domain in epoRT may alter the ability of the receptor to form homo- and heterodimers, a property which is believed to be necessary for signal transduction. This may affect the second messenger systems that are stimulated by the two receptor forms, and thus, produce distinct responses (Watowich *et al*, 1992; D'Andrea *et al*, 1989b).

As well blocking apoptosis and acting as a mitogen, it is likely that epo can also independently direct the transcription of erythroid-specific genes, since highly proliferative erythroleukaemia cell lines that do not undergo apoptosis can be induced to express these genes when treated with epo (Weiss *et al*, 1989). This may involve up-regulation of *GATA-1* gene expression, which, interestingly, is able to enhance the expression of the *epoR* gene, thus forming a positive feedback loop to enhance

differentiation induction by epo (Chiba *et al*, 1991; Zon *et al*, 1991). It is not known exactly how this loop is broken later in erythroid differentiation when *epoR* expression is down-regulated. However, it has been proposed that the epo-induced increase in the level of GATA-1 protein, from the low basal levels seen in earlier progenitor cells, is necessary to switch on erythroid-specific genes (Chiba *et al*, 1991; Sposi *et al*, 1992).

Surprisingly, numerous other cell types outwith the erythroid lineage express epo receptors. Multipotential haemopoietic stem cells express low levels of *epoR* mRNA, detectable only by PCR, and it is debatable as to whether a functional receptor is translated and translocated to the membrane (Heberlein *et al*, 1992). Indeed, it has been proposed that a major control point in the cell surface expression of epoR may be at the level of translocation to the membrane (Migliaccio *et al*, 1991). However, functional, cell-surface epo receptors have been detected on megakaryocytes (Fraser *et al*, 1989), B-lymphocytes (Kimata *et al*, 1991) and endothelial cells (Anagnostou *et al*, 1990) and treatment with epo produces distinct responses, the physiological role of which is not understood.

1.3.2: The haemopoietic microenvironment and erythropoiesis.

Endocrine stimulation of erythroid progenitors by epo is, without question, the key regulatory process that determines the number of mature erythrocytes produced in an adult mouse. However, epo alone is not sufficient to support long-term erythropoiesis and other regulatory molecules must be essential for the lineage commitment, survival, proliferation and maturation of early progenitor cells that eventually give rise to the epo-responsive CFU-E cell population. Many factors have been identified that stimulate early progenitor cells, but their role in normal steady-state erythropoiesis must be considered in the context of the structural organisation of the haemopoietic compartments. In the marrow, haemopoietic cells from all the different cell lineages are intimately associated with stromal tissue, a network of cells and extracellular matrix which not only physically supports the haemopoietic cells but

is also crucial in regulating their maturation (reviewed in Chabannon and Torok-Storb, 1992, and Quesenberry, 1992). The bone marrow stroma contains several cell types including fibroblasts, macrophages, endothelial cells and T and B lymphocytes. It has been known for many years that haemopoietic cells from different lineages proliferate in defined zones of the bone marrow (Lord *et al*, 1975; Lambertsen and Weiss, 1984). These observations led to the proposal that the stroma itself is compartmentalized into a series of haemopoietic microenvironments. Each is thought to commit its resident pluripotent stem cell to a particular lineage by stimulating the stem cell and progenitor cells with a cocktail of cytokines and regulatory molecules produced locally by distinct stromal cells (Trentin, 1970).

Due to the highly structured nature of the stroma it has been difficult to initiate *in vitro* studies that accurately reflect the *in vivo* situation. However, much of what we do know concerning the haemopoietic microenvironment has come from investigating long-term bone marrow cultures (LTBMC).

1.3.3: Long-term bone marrow culture.

When cultured in the appropriate conditions, bone marrow cells give rise to a complex adherent layer of stromal cells into which immature progenitor cells seed where they proliferate and differentiate into mature myeloid blood cells in the absence of exogenous factors (reviewed in Quesenberry, 1992). Modifying the culture conditions results in the production of pre B and B cells (Whitlock and Witte, 1982), probably as a result of changes in the constituents of the stroma and/or cytokine production (Johnson and Dorshkind, 1986). Others have shown that T cell precursors can also be generated (Touw and Lowenberg, 1984); thus by slight variations in the culture conditions all the mature cells of the blood can be formed. In murine LTBMCs, production of haemopoietic cells continues for up to a year, suggesting that multipotential stem cells with a high proliferative capacity are supported by the adherent layer.

The presence of the adherent stromal cells is absolutely required for long-term haemopoiesis *in vivo*. Furthermore, direct contact (Dexter *et al*, 1977), or at least extremely close association (Verfaille, 1992), is also required, implying that cytokines and other regulatory molecules are concentrated locally on the surface of the stroma. BFU-E cells are identifiable in these cultures, but no mature erythrocytes are produced unless erythropoietin is added (Eliason *et al*, 1979; Tsai *et al*, 1986). Thus, with respect to erythropoiesis, all factors required to generate and support BFU-E cells are present in the adherent cell layer in LTBMCs, which probably quite accurately reflects the importance of stromal cells in erythropoiesis *in vivo*.

1.3.4: Erythropoietin and the microenvironment.

Several observations suggest that epo may not be acting solely as an endocrine factor, but may also be produced locally by cells of the haemopoietic organs. First, structures called erythroblastic islands have been identified *in vivo*, and also *in vitro* in LTBMCs. Originally reported by Bessis (1958), these consist of maturing erythroblasts clustered around a central macrophage, with more mature cells more distal. Evidence has been presented from *in situ* hybridisation experiments, which suggests that a subset of macrophages in LTBMCs and *in vivo* can express the *epo* gene, implying that erythropoiesis can be regulated by short range, or cell-to-cell, interactions within the bone marrow between epo and its receptor (Rich, 1988; Vogt *et al*, 1991). This result has been supported by sensitive PCR experiments that have enabled detection of *epo* mRNA in bone marrow cells. Recent evidence suggests that cells other than macrophages may also express the *epo* gene in the haemopoietic organs. If bone marrow cells are pre-incubated for 36 hours with antisense *epo* oligonucleotides, there is a decrease in the number of mixed erythroid/non-erythroid colonies formed in subsequent epo/interleukin-3-stimulated 'burst'-forming assays compared to controls containing *epo* sense sequences (Hermine *et al*, 1991). However, the number of colonies consisting entirely of erythroid cells is unaffected by the oligonucleotides. This result suggested that locally-produced epo was essential

for the survival of epo-responsive multipotential myeloid precursor cells, but not for BFU-E cells, during the pre-incubation stage. Surprisingly, inclusion of epo protein in the antisense pre-incubation did not prevent the inhibition of colony formation, which the authors suggest is evidence that epo and its receptor are produced by the same cell and interact internally, analogous to the gp55:epoR interaction seen in Friend virus-infected erythroid progenitors (see section 2.3.2) (Hermine *et al*, 1991; Yoshimura *et al*, 1990a). Nevertheless, antisense experiments are always subject to problems of specificity: it is possible that the antisense *epo* oligonucleotides used in these experiments affect the expression of other genes involved in multipotential progenitor survival and not just the *epo* gene. Thus, whilst the role epo plays in the stromal microenvironment remains unclear, it seems unlikely that it is acting solely as an endocrine factor.

1.3.5: The role of IL-3 and GM-CSF in erythropoiesis.

Two haemopoietic cytokines, interleukin-3 (IL-3) (Ihle *et al*, 1983; Yokota *et al*, 1984) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Burgess *et al*, 1977; Gasson *et al*, 1984), have a wide variety of effects, stimulating the progenitor cells from all myeloid lineages (reviewed in Arai *et al*, 1990). Use of either of these factors in combination with low levels of epo greatly enhances the number and size of 'bursts' of haemoglobin-containing cells formed from bone marrow cells in clonogenic assays compared to when epo was used alone. Using them both together with epo has an even more dramatic effect, and the concentration of epo used in the experiment can be reduced to near physiological levels (Sonoda *et al*, 1988). Using highly purified blood-borne BFU-E cells, Sawada *et al* (1990) concluded that developing erythroid cells are initially IL-3/GM-CSF-responsive, but that this is then lost as the cells mature into the epo-dependent CFU-E stage. The pleiotropic effect of these two cytokines has been demonstrated *in vivo* by administering them to experimental animals and showing a huge expansion of myeloid progenitor cells, including BFU-E and CFU-E (Metcalf *et al*, 1986; Donahue

et al, 1986). Furthermore, transplantation of bone marrow cells, infected with a retrovirus over-expressing the *IL-3* gene, into lethally-irradiated syngeneic hosts, generates a non-neoplastic myeloproliferative syndrome in mice, again dramatically increasing the BFU-E and CFU-E numbers (Chang *et al*, 1989).

However, the role of these factors *in vivo* is a matter of some controversy. Although, GM-CSF protein and mRNA is readily detectable in the stromal cell layer of LTBMCs that have been exposed to radiation (Gualtieri *et al*, 1984; Alberico *et al*, 1987), or interleukin-1 (IL-1) (a multifunctional inflammatory mediator) (Yang *et al*, 1988), it has been very difficult to detect in unstimulated stroma. However, using highly sensitive PCR protocols, Kittler *et al* (1992) have shown that *IL-3* and *GM-CSF* mRNA are present at very low levels in unstimulated stromal cells. These levels of expression, however, would suggest that haemopoietic cells are only ever exposed to suboptimal concentrations of these factors in the medium. However, LTBMC stroma is able to support the growth of factor-dependent haemopoietic cell lines, an effect that is abrogated by the inclusion of antibodies directed against IL-3 or GM-CSF in the culture (Alberico *et al*, 1987; Kittler *et al*, 1992). Furthermore, recent work implies that components of the extracellular matrix (ECM) may be involved in binding and presenting growth factors to target cells such that their effective concentration locally is increased dramatically. Gordon *et al* (1987) and Roberts *et al* (1988) have shown that sulphated glycosaminoglycans, the major component of which is heparan sulphate, adsorb both GM-CSF and IL-3, retaining them in an active form. In LTBMCs this may explain the necessity for the extremely close association between haemopoietic cells and the stroma in blood cell development (Verfaille, 1992). *In vivo*, this could create microenvironmental niches that direct the maturation of specific cell types.

Despite this evidence, it is still debatable whether IL-3 and GM-CSF are involved in erythropoiesis under normal steady-state conditions. Zipori (1990) and Arai *et al* (1990) have argued that the sole function of these cytokines is as components of 'emergency' haemopoiesis associated with immune defence. IL-3 and

GM-CSF are produced by activated T cells (Arai *et al*, 1990) and support the proliferation of mature haemopoietic cells, such as neutrophils and macrophages (Gasson *et al*, 1984; Vairo and Hamilton, 1991). Their effect on progenitor cells may also be important in supplementing mature blood cells at the sites of infection by stimulating the proliferation of blood-borne progenitors. Infection would also be likely to induce IL-3 and GM-CSF production by certain immune-responsive cells in the bone marrow, and thereby increase blood cell production; alternatively, other factors, such as IL-1, circulating in the blood as a result of infection could stimulate their production from bone marrow stromal cells (Arai *et al*, 1990; Yang *et al*, 1988).

A recent report suggests that GM-CSF may even indirectly down-regulate erythropoiesis in some situations (Wang *et al*, 1992a). Burst formation by BFU-E cells is reduced in GM-CSF/epo-containing medium if macrophages are added. GM-CSF induces the secretion of TNF-α by stromal cells and macrophages. TNF-α is a negative regulator of erythropoiesis (see section 1.3.8.5 below), and is able to negate the positive stimulatory effect of GM-CSF on erythroid progenitors. Combining this with the known stimulation of other myeloid lineages by GM-CSF (that are relatively insensitive to TNF-α), *in vivo*, there will be a shift in the lineage-commitment of multipotent myeloid precursor cells away from the erythroid lineage at times when GM-CSF production is high, and there are a large number of macrophages present. This is indeed seen during chronic infection, with anaemia often accompanying increases in macrophage production.

1.3.6: Stem cell factor; an essential component of the haemopoietic microenvironment.

1.3.6.1: The *white-spotting* and *Steel* loci.

The importance of the stromal microenvironment in the development, maintenance and regulation of haemopoiesis is illustrated by *Steel* (*Sl*) and *white-spotting* (*W*) mutant mice. Mutations at a single autosomal locus in these animals results in altered coat colouration, defective gonad development, varying degrees of

anaemia and a reduction in the number of mast cells (reviewed in Russell, 1979). Numerous distinct alleles have been identified (e.g. *Sl*, Sl^d, *W*, and W^{42}) which, in a homozygous state, generally cause embryonic lethality due to severe anaemia. Heterozygotes (e.g. *Sl*/+) or compound heterozygotes (Sl/Sl^d) are still viable and can reach maturity, but still exhibit the phenotype to varying degrees. Despite the similarities between the *Sl* and *W* mice, two distinct cell populations are affected. This was demonstrated *in vivo* by a series of experiments performed by McCulloch and co-workers (McCulloch *et al*, 1964, 1965): these showed that haemopoietic stem cells from a *Sl*, but not a *W*, heterozygote could rescue a lethally-irradiated normal mouse, whilst normal haemopoietic cells could cure a *W* mutant, but not a *Sl*, mouse. Furthermore, stromal cell feeder layers generated from a *Sl* mouse are unable to support long-term stem cell growth in LTBMCs, whereas stromal cells from a *W* mouse are unaffected by their mutation (Dexter and Moore, 1977). Thus, in *Sl* mice the microenvironment is deficient in providing support for progenitor cells of the haemopoietic system, whilst in *W* mutants it is the progenitors themselves that are defective.

1.3.6.2: The *white-spotting* locus encodes the c-kit receptor.

The products of these two loci have now been identified. The *W* locus on chromosome 5 codes for the c-kit protein, a tyrosine kinase receptor containing five extracellular immunoglobulin-like repeats that is closely related to the receptors for platelet-derived growth factor and colony-stimulating factor-1 (Geissler *et al*, 1988; Chabot *et al*, 1988; Qiu *et al*, 1988). All *W* mutations result in a marked reduction in the kinase activity of the receptor, either by directly reducing the level of *c-kit* mRNA or by the presence of inactivating point mutations in the *c-kit* coding region (Nocka *et al*, 1989, 1990a; Tan *et al*, 1990; Reith *et al*, 1991; Dubreuil *et al*, 1990). As expected, expression of functional c-kit receptors rescues the defect of *W* mutant cells (Alexander *et al*, 1991), whilst injection of anti-kit antibodies into normal wild-type mice mimics, at least to some extent, the effect of mutation within the *c-kit* gene seen

in *W* mice (Ogawa *et al*, 1991). Surprisingly, *W* mutants are known that have point mutations in *c-kit* but only exhibit some of the phenotypic abnormalities described above (Reith *et al*, 1990). Furthermore, *c-kit* mutations are present in humans suffering from piebaldism, a genetic disease which alters skin pigmentation but has no effect on haemopoiesis and gonad development (Giebel and Spritz, 1991; Fleischman *et al*, 1991). Thus, some *c-kit* mutations only affect certain cell types implying tissue-specific roles for certain domains in the c-kit protein. Interestingly, in contrast to the *W* mutations, other abnormalities in *c-kit* can be oncogenic; the HZ4 feline sarcoma virus contains a transduced cellular gene which encodes a mutated, truncated version of the receptor, termed v-kit (Besmer *et al*, 1986), which is thought to possess constitutive tyrosine kinase activity.

1.3.6.3: Stem cell factor, a ligand for c-kit.

The *Steel* locus on chromosome 10 encodes stem cell factor (SCF), a protein that acts as a ligand for c-kit, binding via the three amino-terminal immunoglobulin-like repeats in the receptor: SCF is also referred to as mast cell growth factor, kit ligand and *Steel* factor (Huang *et al*, 1990; Anderson *et al*, 1990; Nocka *et al*, 1990b; Williams *et al*, 1990a; Copeland *et al*, 1990; Zsebo *et al*, 1990a, 1990b; Martin *et al*, 1990; reviewed in Witte, 1990; Blechman *et al*, 1993; Lev *et al*, 1993). Expectedly, infusion of *Sl* mice with SCF produces a marked reduction in the severity of their anaemia and increases their mast cell number (Zsebo *et al*, 1990b). As is the case with a number of other cytokines such as transforming growth factor-α (TGF-α) (reviewed by Massague, 1990), SCF is produced as a transmembrane (TM), as well as a soluble, form (Flanagan and Leder, 1990; Huang *et al*, 1990, 1992). Alternative splicing of the *SCF* mRNA determines if it is retained in the membrane or if a proteolytic cleavage site is included that can be digested to efficiently release soluble SCF. The expression of these two forms is regulated in a tissue-specific manner, and cleavage can be induced by extracellular stimuli, an effect, which in the case of TGF-α, is mediated by the cytoplasmic tail of the protein (Huang *et al*, 1992; Bosenberg *et*

al, 1992). Both forms of SCF stimulate cell proliferation, but cells expressing TM SCF are able to adhere to c-kit-expressing cells (Flanagan *et al*, 1991).

The importance of TM-SCF *in vivo* has been implied by studying certain *Steel* mutants. Mice homozygous for the *Steel-Dickie* allele (Sl^d) develop to adulthood and show a classic *Steel* phenotype but seem to have no reduction in the amount of *SCF* mRNA produced. However, these animals produce no TM SCF as a result of a deletion that removes the TM and intracellular domains of the gene, but still produce biologically-active soluble SCF (Flanagan *et al*, 1991; Brannan *et al*, 1991; Huang *et al*, 1992). This is highly suggestive that direct cell-cell interactions mediated by TM SCF and c-kit are necessary for the development of the murine haemopoietic system. It is possible that these interactions are required to direct stem cells and progenitors from the yolk sac to the liver, and then to the bone marrow and spleen, during embryogenesis. It is at the time of these migrations that the *Sl/W* phenotype becomes apparent, and indeed, SCF is expressed in increasing amounts along the appropriate migratory pathways to the homing sites (Matsui *et al*, 1990; Keshet *et al*, 1991), whilst c-kit is present on the migrating cells (Orr-Urteger *et al*, 1990; Keshet *et al*, 1991). Furthermore, it has been proposed that the TM SCF has different stimulatory properties from the soluble form. Dolci and her co-workers (1991) showed that soluble SCF is relatively limited in its ability to support the long-term survival of primordial germ cells in culture whilst the TM form was much more proficient. Steel *et al* (1992) have suggested that the same may be true for embryonic melanoblasts. Furthermore, TM SCF not only establishes cell-cell adhesion in LTBMCs but also maintains haemopoietic progenitors in culture much longer than soluble SCF (Toksoz *et al*, 1992). Differences in a cells response to soluble versus TM growth factors could occur through several mechanisms including (a) changes in receptor internalisation (Blume-Jensen *et al*, 1991), (b) generation of different intracellular messages, or (c) establishing other stimulatory interactions between receptors and ligands such as IL-3, GM-CSF or components of the ECM as a result of juxtaposing the two cell types.

There is some evidence that the cytoplasmic region of SCF may play an important role *in vivo*. The *Steel*^{17H} allele is only a weak mutant: homozygotes survive and only exhibit mild anaemia and coat colour changes, and intriguingly, only male animals are sterile. This allele has lost 68 nucleotides from its mRNA as a consequence of a splicing defect, resulting in the loss of 23 amino acids and a frame shift in the cytoplasmic region of the protein. Whether the phenotype affects either the processing of this protein to the membrane, or its cleavage to produce the soluble form, is uncertain, but it raises important questions concerning signalling into stromal cells, and the sex-specificity of TM SCF (Brannan *et al*, 1992).

The *Sl/W* phenotype in embryonic-lethal homozygotes appears to be a consequence of a block in cell survival and migration during development. The heterozygotes phenotype could be interpreted as a reduction in the number of stem cells reaching the target organs. However, in adult mice, SCF still plays a central role in haemopoiesis (reviewed in Broxmeyer *et al*, 1991a). Normal haemopoietic cells are unable to rescue *Sl* heterozygotes, whilst injection of normal adult mice with anti-kit antibodies causes a rapid reduction in the number of myeloid progenitor cells, including BFU-E and CFU-E, followed eventually by loss of the mature cells from these lineages (Ogawa *et al*, 1991). Furthermore, antisense *c-kit* oligomers drastically inhibit burst formation by BFU-E cells (Ratajczak *et al*, 1992). Thus, myeloid and erythroid progenitor cells present in adult animals appear to be dependent on SCF for survival. This has been supported using *in vitro* clonogenic assays and purified soluble SCF. Alone, SCF has little effect on progenitor cells, although it can induce proliferation and maturation of mast cells (Nocka *et al*, 1990b; Tsai *et al*, 1991a). However in combination with other cytokines, SCF has a potent synergistic effect on progenitors. With GM-CSF or granulocyte colony-stimulating factor (G-CSF) it stimulates the formation of myeloid colonies (Ulich *et al*, 1991). Interleukin-7 (IL-7), a lymphoid-specific cytokine, and SCF combine to stimulate pre-B cells, a surprising result considering that B-lymphopoiesis is unaffected in *W* mice or by anti-kit antibodies *in vivo* (McNiece *et al*, 1991). As would be expected, in combination with

epo, SCF dramatically increases erythroid 'burst' formation (Martin *et al*, 1990; Anderson *et al*, 1990; Nocka *et al*, 1990b; Broxmeyer *et al*, 1991b; Dai *et al*, 1991). Furthermore, multipotent myeloid precursor cells are also highly responsive to SCF (Broxmeyer *et al*, 1991b; Carow *et al*, 1991), although it is still questionable as to whether SCF is able to directly stimulate pluripotent haemopoietic stem cells (Broxmeyer *et al*, 1991a; Wineman *et al*, 1993). In many cases, the concentration of cytokine required to elicit this synergism with SCF is extremely low, comparable to levels seen *in vivo*. Therefore, a model has been proposed in which SCF acts to 'prime' cells to respond to local or circulating factors, such as IL-3 and epo respectively, at the physiologically relevant level (Lowry *et al*, 1992).

Thus, in summary, unlike GM-CSF and IL-3 and many other factors whose involvement in steady-state erythropoiesis has only been inferred by *in vitro* experiments, the results discussed here with the *Sl/W* mutants demonstrate that SCF is an essential component of the haemopoietic microenvironment controlling blood cell production. The different properties of the two forms of the growth factor (either soluble or membrane-anchored) imply that direct cell-cell contact between progenitor cells and the stroma, mediated by TM SCF, may be central to this control.

1.3.7: Integrins and the extracellular matrix.

The ECM of the haemopoietic organs plays a dual role in erythropoiesis. First, it acts as an anchorage site for progenitor cells. CFU-E, and to a lesser extent BFU-E, adhere to fibronectin (Fn) (Weinstein *et al*, 1989), whereas granulocyte progenitors preferentially bind to haemonectin, a separate component of the ECM (Campbell *et al*, 1987). Both are able to bind to collagen type I (Koenigsmann *et al*, 1992). These differences in the adhesive properties of specific progenitor cell populations, may be essential to direct them to distinct microenvironments that permit further maturation. To allow the erythrocytes to enter the blood stream, as the CFU-E mature, the number of Fn receptors decreases and adhesion is lost (Vuillet-Gaugler *et al*, 1990).

The second possible function is as a direct stimulant of cell proliferation and/or survival. For example, Fn induces a modest increase of erythroid 'burst' formation *in vitro*, an effect that may be mediated by signal transduction through the Fn receptor (Werb *et al*, 1989). Indeed, ECM receptor proteins (integrins) are now believed to initiate intracellular signals that influence cell growth, differentiation and morphology (reviewed in Damsky and Werb, 1992). The Fn receptor is not the only integrin expressed during erythropoiesis: many other adhesion molecules have been detected on erythroid progenitor cells and their expression is modified as they mature (Papayannopoulou and Brice, 1992). The exact role of these interactions during erythropoiesis remains to be elucidated, but it seems likely that they affect the processes of maturation and proliferation as part of an inductive microenvironment for erythropoiesis.

The ECM also plays an important indirect role in haemopoiesis. As discussed above, GM-CSF and IL-3 bind to heparan sulphate where they are retained in an active form. Other studies suggest that interaction with the ECM is important in the effective presentation of growth factors to cells. For example, basic fibroblast growth factor also binds to heparan sulphate, but unlike IL-3 and GM-CSF, this is necessary to enable it to bind to its high affinity receptor on target cells (Yayon *et al*, 1991). Leukaemia inhibitory factor, on the other hand, can be secreted in two forms from embryonic fibroblasts. One is a soluble molecule whilst the other contains a unique amino-terminus, as a consequence of transcription from an alternative promoter, that causes it to become immobilised on the ECM (Rathjen *et al*, 1990). Alternatively, as for SCF, growth factors can be produced as membrane proteins, which along with the mechanisms discussed here, have the effect of concentrating growth factors on a relatively immobile two-dimensional surface. It seems highly likely that this is essential to restrict regulation of cell growth and development to a small area of the bone marrow and to permit the establishment of many heterogeneous lineage-specific niches.

1.3.8: Other erythropoietic regulators.

Several other molecules are able to influence erythrocyte production, by stimulating or inhibiting cell proliferation, maturation or survival. A number of these that are believed to exert a direct effect on erythroid progenitors are discussed below, although their exact role in the context of the haemopoietic microenvironment is often a matter of some controversy.

1.3.8.1: Erythroid-potentiating activity.

Erythroid-potentiating activity (EPA) was originally purified from the conditioned-medium of a T-lymphoblast cell line which had the ability to synergise with epo to induce erythroid 'burst' formation *in vitro* (Westbrook *et al*, 1984; Gasson *et al*, 1985). *In vivo* administration of recombinant protein to anaemic mice causes an increase in the number of erythroid precursors in the spleen, and erythrocytes in the blood (Niskanen *et al*, 1988). EPA is expressed in the haemopoietic organs and is produced in an active form by stromal and erythroleukaemia cells (Hayakawa *et al*, 1990; Avalos *et al*, 1988). EPA signals via specific receptor molecules on progenitor cells to up-regulate erythropoietin receptor expression (Fraser *et al*, 1988). Surprisingly, EPA is identical to a member of the family of tissue inhibitors of metalloproteinases-1, which as their name suggests were isolated due to their ability to prevent ECM degradation by metalloproteinases (Docherty *et al*, 1988). In view of the importance of the ECM in haemopoiesis, this molecule could also affect erythropoiesis indirectly through a local restructuring of the microenvironment.

1.3.8.2: Burst-promoting activity (BPA).

Daniak and Cohen (1982) identified an activity in conditioned-medium from B-lymphocytes that acted synergistically with epo to induce erythroid 'burst' formation *in vitro*. Although the gene for this protein has yet to be cloned, it was shown that the activity is caused by an integral membrane protein, termed BPA, present in exfoliated vesicles from the B-cells interacting with early erythroid

progenitors (Feldman *et al*, 1987). This represents a unique mechanism whereby the close proximity of two cell types, but not necessarily direct contact, is sufficient to exchange growth regulatory signals present in the membranes. As with all these factors, the exact role of BPA *in vivo* is unknown, but its association with an immune cell suggests it may be involved in 'emergency' haemopoiesis induced on infection. However, B-cells are also believed to be a component of the haemopoietic stroma (Quesenberry, 1992) and thus, BPA may be involved in establishing an erythropoietic microenvironment.

1.3.8.3: Steroids: thyroid hormone and retinoic acid.

Anaemia is frequently a complication of hypothyroidism in humans, responding only to thyroid hormone (T3) replacement therapy (Fein and Rivlin, 1975). This hormone stimulates erythropoietin production (Peschle *et al*, 1971), but also appears to have a more direct effect on immature erythroid cells, acting to increase epo/IL-3-induced erythroid 'burst' formation in *in vitro* assays (Dainiak *et al*, 1978). Dainiak and his co-workers later suggested that this was due to T3 inducing the production of erythropoietic growth factors by accessory cells, probably lymphocytes, and not as a direct effect on the erythroid progenitors themselves (Dainiak *et al*, 1986). However, a more detailed recent study (Schroeder *et al*, 1992) using pure populations of chick erythroid cells, suggests that this is probably not a full explanation. These experiments were performed on erythroid progenitors reversibly transformed with a temperature-sensitive (ts) *v-sea* oncogene. At the non-permissive temperature when the cells were no longer transformed, treatment with T3 weakly accelerated the differentiation of the progenitors, but induced premature cell death in more mature cell types. The same phenomenon was also observed in erythroid progenitors isolated by virtue of their ability to grow in TGF-α, suggesting that the ts-*v-sea* oncogene was not involved in the effect.

Interestingly, retinoic acid (RA) produced a similar response to T3 in these experiments, but was much more potent, and furthermore, exhibited a synergistic

effect when used in combination with T3 (Schroeder *et al*, 1992). As in hypothyroid individuals, there is often a correlation between retinol (vitamin A) deficiency and anaemia (Koeffler and Amatruda, 1985). The similarity in the responses elicited by T3 and RA is probably due to the functional similarity of their receptors, both signalling via nearly identical response elements (reviewed in Beato, 1989), whilst it has been proposed that the observed synergism between these two steroids is possibly an indication of heterodimerisation of the two receptors (Schroeder *et al*, 1992; Hudson *et al*, 1990). The importance of these two factors in normal erythropoiesis *in vivo* is supported not only by the correlations between deficiency and anaemia, but also by the oncogenic effect *v-erbA*, a mutated form of the T3 receptor carried by the avian erythroblastosis virus (AEV) (section 2.2.1). As its name suggests AEV induces erythroleukaemias in chickens, with the v-erbA protein preventing RA and T3-mediated signals, blocking the expression of certain erythroid-specific genes, and thus, disrupting differentiation.

1.3.8.4: Activin.

Eto and his co-workers (1987) purified a protein, termed erythroid differentiation factor (EDF), from a phorbol ester-treated monocytic cell line, that was able to induce the differentiation of murine erythroleukaemia cells and enhance the growth of normal erythroid precursors *in vitro* and when administered to mice *in vivo* (Yu *et al*, 1987; Broxmeyer *et al*, 1988; Shiozaki *et al*, 1990a). EDF has also been shown to have a stimulatory effect on megakaryocytic differentiation (Fujimoto *et al*, 1991) and the growth of multipotential progenitor cells (Broxmeyer *et al*, 1988). It has been demonstrated that EDF is identical to activin (Murata *et al*, 1988), a protein that is known to enhance the secretion of follicle-stimulating hormone from pituitary cells (Vale *et al*, 1986; Ling *et al*, 1986) and appears to be involved in determining cell fate in early embryos (reviewed in Wylie, 1990). There are several observations that imply that activin/EDF plays a physiological role in normal steady-state erythropoiesis. First, Yamashita and his colleagues (1992) have demonstrated that

murine bone marrow stromal cells express the gene, and respond to cytokines, like interleukin-1, to increase production. Second, Shiozaki *et al* (1992), who confirmed this observation, went on to show that the protein was produced in an active form by these cells. More importantly, they demonstrated that follistatin (a specific binding protein that blocks activin function) induces a decrease of the erythroid progenitor cell population (BFU-E and CFU-E) when administered to mice. Surprisingly, however, the number of mature erythrocytes produced was unchanged. This implies that activin has a role in controlling steady-state erythropoiesis, but that other factors can compensate if it is inhibited.

1.3.8.5: Negative effect of tumour necrosis factor-α.

Macrophages reduce *in vitro* colony formation by CFU-E cells whilst infusion of macrophages into mice that have been infected with the Friend virus (see section 2.3 below), that have a large increase in CFU-E numbers, results in the reversal of the disease state (Marcelletti and Furmanski, 1978). Indeed, during Friend disease macrophages become infected and their ability to suppress erythropoiesis is lost (Marcelletti and Furmanski, 1979). Furthermore, it is now generally accepted that the anaemia that often accompanies chronic infection, is due to macrophage proliferation (see section 1.3.5). Macrophages release a large number of cytokines, including interleukin-1-α (IL-1-α) which mediates this erythrosuppressive effect by synergising with tumour necrosis factor-α, induced in soluble or membrane bound forms (Massague, 1990) by stromal cells and macrophages in response to IL-1α and GM-CSF (see above) (Furmanski and Johnson, 1990; Wang *et al*, 1992a). IL-1-α release increases at times of infection so shunting haemopoiesis toward the production immune defence cells at the expense of erythrocytes.

1.4: Summary.

Alterations in the level of production of mature red blood cells that are needed to meet the requirements of the animal, are almost exclusively controlled by the

hormone erythropoietin, which enhances the survival of erythroid progenitors already in the process of differentiation. Only at times of infection or blood loss are other growth factors, such as IL-3, GM-CSF and TNF-α, likely to affect erythrocyte production. However, the viability, proliferation and lineage commitment of the progenitors is largely determined by the nature of the local microenvironment which contains a complex combination of growth factors, extracellular matrix components and cell adhesion molecules produced by haemopoietic and stromal cells. The study of these interactions has only been possible using genetically-defective mice (*Steel* and *white-spotting* mutants) and complex *in vitro* systems, like LTBMCs. To further understand the role of the microenvironment, it would be advantageous to generate cell systems in which complexity is reduced by decreasing the number of cell types in the culture, whilst maintaining the physiological dependence on stroma interaction. The availability of these simplified coculture systems would also permit an investigation into the role of stroma in leukaemia: one could hypothesise that a cell able to proliferate outside the haemopoietic microenvironment would have a considerable advantage over its normal counterparts. Identifying genetic alterations which are able to overcome stromal cell-dependence, would not only give an insight into the signal transduction pathways activated by stromal cell interaction, but may show that overcoming the control exerted by the microenvironment can be a crucial part of leukaemic progression.

CHAPTER 2: Models for Erythroleukaemia in Experimental Animals.

2.1: Retrovirally-induced leukaemias: An overview.

Leukaemia can be defined as the uncontrolled proliferation or expansion of haemopoietic cells that lose the ability to differentiate normally to mature blood cells. This implies that the developing leukaemic cell must become insensitive to the

normal signals that regulate its proliferation, survival and maturation. This is dependent on the accumulation of stable genetic mutations that activate proto-oncogenes and disrupt the inhibitory effects of tumour suppressor gene products. In humans, these genetic alterations are either inherited or arise spontaneously, probably to some extent as the consequence of exposure to environmental mutagens and carcinogens throughout life. Many animal model systems have been developed to study tumorigenesis, in which a defined treatment will repeatedly generate malignancies of a specific type. These have been essential in the identification of genes targeted in tumour progression and the definition of their role in disrupting normal growth and development.

Important advances in understanding normal and malignant haemopoiesis have come from the study of murine and avian leukaemia viruses (reviewed in Teich *et al*, 1982). These can be divided into two classes determined by their structure and leukaemogenicity. The first group, termed the acute transforming retroviruses, rapidly induce leukaemias within two to three weeks of infection. They lack the classical *gag-pol-env* genomic structure of a retrovirus since viral genes have been replaced by transduced, mutated cellular genes that are crucial for leukaemogenicity e.g. the avian erythroblastosis and E26 viruses, and Abelson murine leukaemia virus. Loss of viral genes usually results in the virus becoming replication-defective, and inclusion of 'helper' viruses to compensate for the defective functions usually decreases latency. Integration of these viruses into host DNA furthers tumour progression by mutating or activating host genes. The slow transforming retroviruses require a longer incubation period due to the absence of a transduced oncogene. These depend mainly on insertional mutagenesis or recombination with host sequences to generate leukaemias; for example, Friend murine leukaemia virus and avian leukaemia virus. Identification of genes deregulated or inactivated by these integrations has demonstrated the involvement of many known and novel genes in tumour progression (reviewed in Peters, 1990).

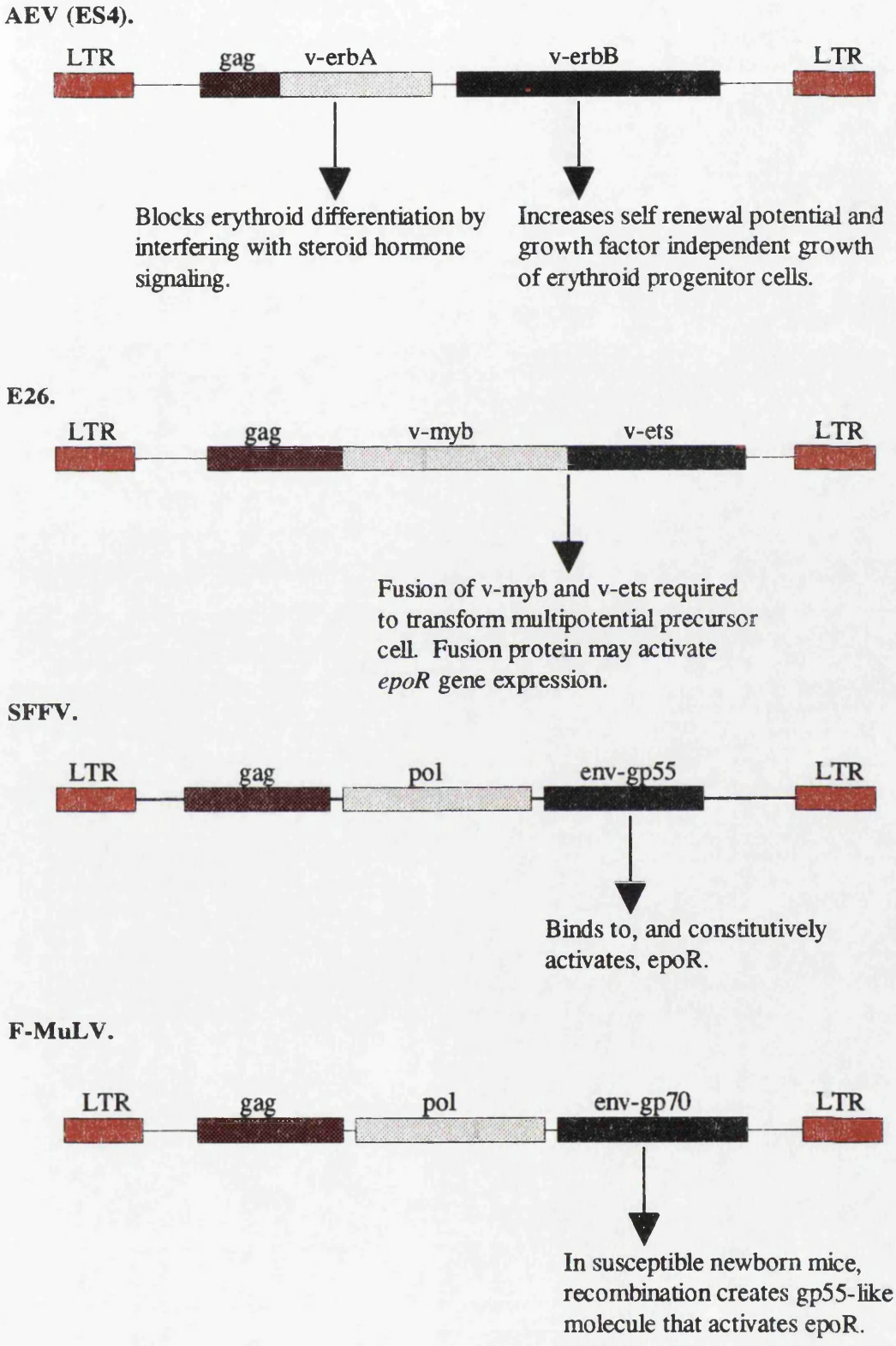
2.2: Avian leukaemia viruses and erythroleukaemias.

Many acute transforming retroviruses have been isolated that are able to induce leukaemias in chickens, the phenotype of which is determined by the transduced host sequences present in the viral genome. Of particular interest to this study are the replication-defective avian erythroblastosis virus strain ES4 (AEV-ES4) and E26, both of which rapidly induce tumours that appear to be derived, at least in part, from cells of the erythroid lineage. Understanding these viruses has led to many important discoveries concerning oncogenes and the mechanisms by which they transform erythroid cells. Both viruses carry transduced sequences derived from two host genes that cooperate by two distinct mechanisms in the development of the leukaemia.

2.2.1: The AEV-ES4 virus.

AEV-ES4, which induces rapid and fatal erythroblastosis in chicks, expresses *v-erbA* and *v-erbB*, oncogenic forms of the thyroid hormone (T3) receptor and epidermal growth factor (EGF) receptor respectively (Sap *et al*, 1986; Weinberger *et al*, 1986; Downward *et al*, 1984): each plays a separate role in generating the phenotype of the leukaemic cells (Figure 2.1). *V-erbB* is capable of increasing the self-renewal potential of early erythroid progenitor cells (BFU-E) in chicks, a property which can also be conferred by infection with viruses carrying a wide variety of activated kinase-type oncogenes (Klinken, 1988). However, v-erbB-transformed cells have also lost their dependence on epo for survival and differentiation, and will mature spontaneously into erythrocytes (reviewed in Hayman and Beug, 1992). Interestingly, this loss of epo-dependence appears to be mediated by mutations in the carboxy-terminus of *v-erbB*, whereas self-renewal can be induced experimentally simply by over-expressing the *c-erbB* gene (Khazaie *et al*, 1988). This often occurs in erythroleukaemias induced with the replication-competent slow-transforming retrovirus avian leukaemia virus, in which *c-erbB* is deregulated as a consequence of insertion of the retrovirus upstream of the host *c-erbB* gene (Fung *et al*, 1983).

Figure 2.1: Structure and function of erythroleukaemogenic retroviruses.



All retroviruses can act as insertional mutagens. Particular insertions will be selected for during tumour progression e.g. activation of *ets* genes in Friend erythroleukaemia. For further description of these viruses, see text.

However, in many of these tumours *c-erbB* mRNA undergoes altered processing to produce proteins lacking the extracellular domain which may be important in the development of the leukaemia (Nilsen *et al*, 1985).

The EGF receptor is expressed on a subset of chicken BFU-E cells and it has been demonstrated that transforming growth factor-α (TGF-α), which signals through the EGF receptor (Lax *et al*, 1988), stimulates the self-renewal of these cells (Pain *et al*, 1991). Thus, v-erbB will stimulate this signalling pathway in the absence of the growth factor. However, EGF receptors have not been detected on mammalian haemopoietic cells (von Ruden and Wagner, 1988). Nevertheless, *v-erbB* is still able to transform murine BFU-E cells (Miller *et al*, 1990); it also induces hyperproliferation of mast cells and myeloid progenitors that eventually leads to myeloid leukaemia (von Ruden *et al*, 1992). In view of these observations, it is interesting to note that (a) cell types affected by *v-erbB* expression in the mouse are all SCF-responsive, and (b) c-kit and the EGF receptor are both kinase-type receptors that share common signal transduction molecules (von Ruden *et al*, 1992). Therefore, in mice, the v-erbB protein could possibly encourage SCF-independent growth of haemopoietic progenitors by constitutive activation of signal pathways used by SCF. However, there have been no reports implicating the EGF receptor in mammalian haemopoietic malignancies.

V-erbB is capable of inducing erythroleukaemias in chicks in the absence of *v-erbA* (Frykberg *et al*, 1983; Yamamoto *et al*, 1983). However, disease latency is reduced significantly if *v-erbA* is included in the virus. This oncogene appears to block the spontaneous differentiation of *v-erbB*-transformed erythroid cells by preventing the expression of erythroid specific genes (Schroeder *et al*, 1990; Fuerstenberg *et al*, 1992). This effect is thought to involve the dominant-negative inhibition of steroid hormone-mediated differentiation signals, in particular, those signalling through the retinoic acid receptor (see section 1.3.8.3 above) (Sharif and Privalsky, 1991; Desbois *et al*, 1991). The mutations in the gene prevent its product from transactivating target genes in the presence of T3, and moreover, by competing

for recognition sequences in promoters and/or enhancers, prevent c-erbA and the retinoic acid receptor from transactivating their target genes.

2.2.2: Fusion of *myb* and *ets* in the E26 retrovirus.

The E26 retrovirus generates leukaemias that exhibit both a myeloid and an erythroid component due to transformation of a precursor cell that has potential to differentiate along the myeloid or erythroid lineages (Graf *et al*, 1992). This virus has an unusual tripartite genomic structure in which viral *gag* sequences are fused to a truncated *c-myb* gene (*v-myb*), which in turn is fused to a sequence termed the *E*26 *t*ransformation-*s*pecific region, or *v-ets* (Figure 2.1) (Nunn *et al*, 1983; Leprince *et al*, 1983), with both *v-ets* and *v-myb* being derived from cellular transcription factors. Alone, the *v-myb* and *v-ets* components of the E26 retrovirus are not leukaemogenic; however, they are able to transform haemopoietic cells *in vitro*, *v-myb* affecting myeloid and erythroid cells whilst *v-ets* only affects erythroid progenitors. When both genes are used together, but as separate proteins, transformation is potentiated but the transformed erythroid cells appear to be more mature than cells expressing the myb-ets fusion protein. Furthermore, this combination is still not leukaemogenic *in vivo* (Nunn and Hunter, 1989; Metz and Graf, 1991a). Only when the two genes are physically fused in a way similar to that found in the original virus is leukaemogenicity restored (Metz and Graf, 1991b). Exactly why fusion of the two parts is necessary is unclear, but it seems likely that each component in some way alters the properties of the other. This would create a molecule that has a unique DNA binding specificity, and possibly has modified interactions with regulatory molecules, such that it is able to control the transcription of a distinct set of genes essential in the transformation of the multipotent progenitor cell.

An artificial murine retrovirus, ME26, which contains the *gag-myb-ets* fusion gene from E26 in an Abelson murine leukaemia virus-derived vector, can, like E26, transform multipotential progenitors and induce erythroid and myeloid leukaemias in newborn, but not adult, mice (Yuan *et al*, 1988). Erythroleukaemic cells from these

animals could be readily established as permanent epo-dependent cell lines, which looked considerably more immature than leukaemic cells from a Friend virus-infected mouse (see section 2.3 below). Ruscetti *et al* (1992) suggested that these cells more closely resembled multipotential precursors than erythroid-specific progenitors, and proposed that ME26 may be inducing them to become epo-responsive. Consistent with this idea, they showed that when an IL-3-dependent murine cell line, FDCP-2, was infected with ME26 they could grow in the presence of epo or IL-3: uninfected or F-MuLV-infected control cells grew only in IL-3. Co-transfection experiments performed by Ruscetti's group in FDCP-2 cells imply that this ME26-specific effect is mediated by the *GATA-1* gene, the expression of which is up-regulated by the gag-myb-ets fusion protein (Aurigemma *et al*, 1992). This may be a direct effect on the *GATA-1* promoter which contains binding sites that may be recognised by myb and ets proteins (Nicolis *et al*, 1991). GATA-1 is able to stimulate transcription of the *epoR* gene through GATA-1 sites upstream of the gene, an effect that is potentiated by interaction of the myb-ets fusion protein with the *epoR* promoter. Increased epoR protein in immature cell types may mediate an epo-induced hyperproliferative state, that could ultimately develop into an erythroleukaemia (Aurigemma *et al*, 1992). Although this is an attractive hypothesis, it is unlikely that it fully explains the leukaemogenicity of E26 or ME26. For instance, ME26 infection acts as a mitogenic stimuli to fibroblasts in the absence of growth factors and without activating *epoR* expression, presumably as a result of activating other target genes (Yuan *et al*, 1988; Aurigemma *et al*, 1992).

2.3: Friend virus-induced erythroleukaemias.

2.3.1: Rapid induction of erythroblastosis in Friend virus-infected mice.

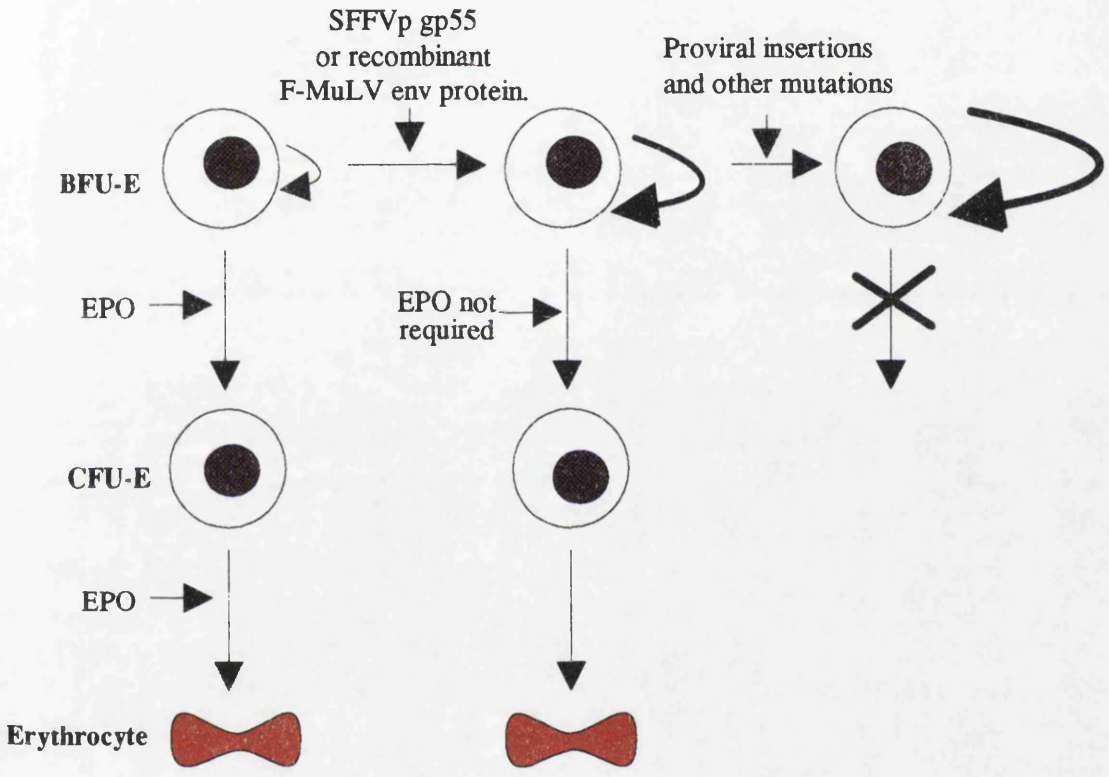
Few of the murine retroviruses induce erythroleukaemias; most affect lymphoid cells or other myeloid lineages. However, the Friend virus (FV) specifically transforms erythroid progenitor cells (for review, see Ben-David and Bernstein, 1991; Kabat, 1989). The original isolate (FV-A) was obtained from a cell-

free spleen extract from an adult mouse that had developed erythroleukaemia following inoculation with Ehrlich mouse carcinoma cells (Friend, 1957). Injection of this extract into other susceptible hosts resulted in a rapid enlargement of the spleen (splenomegaly), and often the liver (hepatomegaly), and induced anaemia due to the large numbers of undifferentiated erythroid progenitor cells in the blood (Tambourin *et al*, 1979). It is likely that most haemopoietic cells become infected by virus, but it is only the epo-responsive erythroid progenitor cells i.e. the CFU-E and late BFU-E populations, that are affected. However, this may be potentiated by FV infection of macrophages preventing TNF-α-mediated erythrosuppression (Marcelletti and Furmanski, 1979). Rauscher virus, an independently-isolated virus from mice injected with ascites tumour cells, is very similar in structure to FV-A. It induces a similar disease, although late after infection leukaemic lymphoid cells are often detectable (Rauscher, 1962; Teich *et al*, 1982). Derivatives of FV-A, termed FV-P, were found to induce splenomegaly characterised by polycythaemia (an over-production of red blood cells), rather than anaemia (Mirand, 1967; Sassa *et al*, 1968). Cells infected by FV-P have completely lost their dependence on epo for growth and differentiation (Mirand, 1967), whereas FV-A is unable to induce total epo-independence and requires additional exogenous growth factor to terminally differentiate. *In vivo*, the concentration of epo is insufficient to cope with the excessive expansion of the erythroid progenitor compartment induced on FV-A infection, and progenitors are released into the blood in an undifferentiated state.

Thus, the early stages of Friend disease are characterised by a polyclonal expansion of erythroid progenitor cells that still differentiate and whose self-renewal capacity is limited (Figure 2.1). Only by replication and re-infection can the disease be maintained. It is only late in the disease when truly leukaemic cells evolve that are blocked in differentiation and have become immortalised. The identification and molecular analysis of these cells will be discussed at greater length below.

Numerous genetic loci control susceptibility to FV infection and seem to act by either blocking replication (e.g the *Fv-1* locus (Pryciak and Varmus, 1992)) or by

Figure 2.2: Multistage Friend erythroleukaemia.
(see text for further details).



	Normal erythropoiesis.	**Polyclonal proliferation.**	**Leukaemic cells.**
Probable cause.	Growth factor stimulation.	Mutant viral env proteins activate the epo receptor.	Deregulated *ets* expression. Loss of wild-type *p53*.
Effect.	Epo-dependent maturation.	Increased proliferation. Epo-independent maturation. Non-tumorigenic.	Immortalised. Growth factor-independent. Maturation arrested. Tumorigenic, *in vivo*.

reducing the number of erythroid progenitor cells available for infection. For example, the *Fv-2* locus controls the expression of a secreted protein, called the negative regulatory protein, that dramatically reduces the number of cycling BFU-E cells, without affecting progenitors from other lineages or, surprisingly, the number of mature erythrocytes released into the blood (Axelrad *et al*, 1981; Del Rizzo *et al*, 1988). Similarly, *Steel* and *W* mutant mice, that have a paucity of haemopoietic progenitor cells (see above), are also resistant to FV infection.

2.3.2: The Friend virus and its oncogene.

Both FV-A and FV-P are a mix of two types of viruses: a replication-defective spleen focus-forming virus (SFFV), and, to allow propagation of the infection, a replication-competent Friend murine leukaemia virus (F-MuLV) (Figure 2.1) (Kabat, 1989). F-MuLV is dispensable if large SFFV titres are used, as the need for replication and re-infection is no longer required (Wolff and Ruscetti, 1985; Wolff *et al*, 1986). Furthermore, transgenic mice harbouring the SFFV genome develop erythroid neoplasms (Aizawa *et al*, 1990). Thus, SFFV is able to act as an acute-transforming retrovirus, although surprisingly it does not contain a transduced cellular gene. Instead, it contains a rearranged *envelope* (*env*) gene that has lost its packaging properties (explaining the requirement for a helper-virus), but is essential for the erythroblastosis in the initial stage of the Friend disease (Figures 2.1 and 2.2) (Wolff and Ruscetti, 1988). Transgenic mice expressing this gene, termed *gp55*, develop erythroleukaemia as readily as those expressing the whole virus (Aizawa *et al*, 1990). Gp55 contains an extracytoplasmic region derived from the endogenous Friend mink cell focus-forming (MCF) viral env protein and a transmembrane domain from the env protein of F-MuLV. The cleavage site found between the gp70 and p15(E) portions of other retroviral env proteins is missing from the *gp55* gene, and thus, it codes for a membrane-spanning fusion protein. Furthermore, a single base insertion in the p15(E)-derived portion causes a shift in the reading frame that truncates the gp55 protein due to premature termination. The deletion and base insertion are found

in the *gp55* genes from all SFFV isolates, implying a functional importance for its leukaemogenicity (Clark and Mak, 1983). Unlike the env proteins from F-MuLV, the gp55 protein is processed inefficiently and 95% is retained in the endoplasmic reticulum. It is here that gp55 interacts with the epo receptor, altering its metabolism and, more importantly, inducing a proliferative signal in the absence of epo (Yoshimura *et al*, 1990a). However, Ferro *et al* (1993) have produced data which contradicts this conclusion to some extent, in that only SFFV-infected cells which transport gp55 to the cell surface can become epo-independent. Nevertheless, the gp55/epoR interaction has been effectively confirmed by two experiments. Ruscetti *et al* (1990) were able to generate factor-independence in an epo-dependent cell line, HCD-57, simply by transfecting in the *gp55* gene from the FV-P strain. Similarly, Li and co-workers (1990) generated factor-independence in the IL-3-dependent lymphoid cell line, Ba/F3, by co-transfecting vectors expressing the *epoR* gene and *gp55* from FV-P.

The interaction between gp55 and epoR is highly specific: gp55 shows no ability to complex with other closely-related growth factor receptors, such as the IL-3 receptor (IL-3R). The interaction with epoR appears to be via an extracellular domain on the receptor distinct from the epo binding site, and also via the membrane-spanning domain (Zon *et al*, 1992; D'Andrea, 1992). This has been demonstrated using two chimeric IL-3R/epoR fusion proteins, that contained either the extracytoplasmic and transmembrane (TM) domains of epoR (chimaera A), or just the extracytoplasmic domain (chimaera B). Both bind to gp55, but only chimaera A activates growth factor-independent growth of the Ba/F3 cell line when co-expressed with the *gp55* gene. However, both are able to induce epo-dependent growth. This suggests that, although epo only requires the extracytoplasmic domain, activation of epoR by gp55 requires interaction via the TM domain. This has been confirmed by similar experiments with chimeric gp55 proteins which designated the TM region as crucial for its leukaemogenicity (Chung *et al*, 1989; Srinivas *et al*, 1991). Interestingly, different FV variants that exhibit altered host ranges and pathology

from FV-P, have alterations in the TM portion of their gp55 protein. For example, the partial epo-dependence of FV-A infected cells is the result of small changes in the sequence of gp55 which presumably have subtle affects on its interaction with the epo receptor (Ruscetti and Wolff, 1985; Wolff *et al*, 1985). Furthermore, the ability of certain FV strains to infect *Fv-2* resistant mice is again a consequence of *gp55* sequence alterations; in particular, a deletion near the transmembrane domain is invariably found in these strains (Majumdar *et al*, 1992). This implies that the negative regulatory protein, whose expression is regulated by the *Fv-2* locus (see section 2.3.1), may in some way modify signal transduction originating from the epo receptor, which certain gp55 molecules are able to bypass.

The high degree of specificity in binding between gp55 and epoR accounts, at least in part, for the erythroid specificity of SFFV. However, the LTR of this virus appears to only be active in erythroid cells and may contribute to the narrow host range (Ruscetti and Wolff, 1984). Activation of growth factor receptors by retroviral env proteins may not be restricted to SFFV. D'Andrea (1992) has proposed that similar interactions may be involved in the leukaemogenicity of other retroviruses. For example, the IL-2 receptor is activated when it is co-expressed with the *env* genes of the Friend MCF and Moloney MCF viruses, although IL-2 receptor/env protein complexes have not been detected (Li and Baltimore, 1991). Furthermore, mutant viral proteins are thought to be involved in causing immunodeficiency and neuronal diseases, which in some cases are dependent on interactions between the mutant viral protein and cell surface receptors (Aziz *et al*, 1989; Paquette *et al*, 1989)

2.3.3: F-MuLV: not just a 'helper'.

The major role of F-MuLV in FV is to act as a 'helper' virus, potentiating SFFV infection and mutating host genes by proviral insertion. However, infection of susceptible strains of *newborn* mice with F-MuLV alone is able to induce a variety of haematological neoplasms, including erythroleukaemias (Troxler and Scolnick, 1978; MacDonald *et al*, 1980; Oliff *et al*, 1981), the type of leukaemia induced depending

on the genetic background of the infected mouse (Shibuya and Mak, 1982; Chesebro *et al*, 1983). Interestingly, these erythroleukaemias invariably have a constitutively active epo receptor. This occurs by one of two mechanisms. First, in many of these neoplasms, recombinant viruses are formed between the exogenous F-MuLV and endogenous retroviral sequences: these viruses express proteins which, like gp55, bind to the epo receptor and transduce a signal in the absence of epo (Figures 2.1 and 2.2) (Ruscetti *et al*, 1981; Chesebro *et al*, 1984; Li and Baltimore, 1991). Second, a cell line from an F-MuLV erythroleukaemia shows constitutive *epo* expression as a result of rearrangement and amplification of the gene (McDonald *et al*, 1987), although it appears that this type of mutation is not often involved in the development of these malignancies.

Thus, viral activation of the epo receptor is intimately linked to the initial stages of Friend disease, irrespective of whether it is induced with FV or F-MuLV. Indeed, it seems likely that constitutive activation of the epo receptor is sufficient for this stage: activation of the epo receptor *in vivo* by other means, for example, by infection with a retroviral vector over-expressing the *epo* gene, or by constitutive epo production in transgenic mice, result in erythroblastosis similar to that seen in FV-infected mice (Hoatlin *et al*, 1990; Semenza *et al*, 1989). Furthermore, a retrovirus carrying a constitutively active, mutant *epoR* gene, is able to confer factor-independence on the Ba/F3 cell line and induce FV-P-like polycythaemia and erythroleukaemia when injected into a mouse (Yoshimura *et al*, 1990b; Longmore and Lodish, 1991), which interestingly, is often accompanied by an increase in granulocyte/macrophage progenitor growth (Pharr *et al*, 1993).

2.3.4: Leukaemic cells arise late in the Friend disease.

Early after infection with FV, or F-MuLV or SFFV alone, the vast majority of the infected erythroid cells have only limited self-renewal capacity and remain capable of terminal differentiation. In semi-solid culture these cells form small colonies of haemoglobinised cells. Furthermore, they will not form tumours if they

are transplanted subcutaneously into syngeneic mice and permanent cell lines can not be established. Only late in the disease (3 weeks or more for FV-P; up to 6 weeks for FV-A; 8 weeks and above for F-MuLV alone) (Shibuya and Mak, 1982; Teich *et al*, 1982) do leukaemic cells arise that exhibit the following characteristics. 1). The ability to form large colonies of undifferentiated cells in semi-solid media, in the absence of epo, that can subsequently be grown in suspension culture as permanent cell lines, without exogenous growth factors or stromal cells (Mager *et al*, 1981a, 1981b). 2). Growth to form tumours *in vivo* by *intra venous* injection, or by transplantation subcutaneously or into the omentum of syngeneic mice, which can then be cultured *in vitro* to form permanent cell lines (Friend and Haddad, 1960; Moreau-Gachelin *et al*, 1981; Wendling *et al*, 1981). 3). Colony formation in the spleens of irradiated normal mice, and in Sl/Sl^d mice that have a defective microenvironment starved of SCF (Mager *et al*, 1980).

Established Friend cell lines are generally unable to differentiate in response to epo, although chemical inducers, such as DMSO (Friend *et al*, 1971), overcome this maturation arrest: this system has been extensively exploited to study erythroid specific gene expression in a pure population of differentiating erythroid cells (reviewed in Harrison, 1982). Thus, in short, in the late stages of the disease individual cells become immortalised, lose their ability to differentiate, and are autonomous of humoral and microenvironmental factors (diagrammatically summarised in Figure 2.2). However, several erythroleukaemia cell lines have been derived that do not fully express this phenotype. For example, HCD-57 cells derived from a NIH Swiss mouse infected at birth with F-MuLV still require epo for growth in culture but are unable to mature to erythrocytes (Spivak *et al*, 1991). The Red 5-1.5 cell line, which came from a Rauscher virus-infected mouse, grows in the absence of exogenous growth factors but will differentiate on the addition of epo (Weiss *et al*, 1989).

Friend erythroleukaemia is, therefore, a multistage disease with a rapid expansion of erythroid progenitors preceding the acquisition of a leukaemic

phenotype (Figure 2.2). This is a common feature of tumour generation with the fully malignant phenotype arising after several non-neoplastic steps. This is typified by human colon carcinogenesis in which the invasive carcinoma is preceded by a non-invasive carcinoma, which itself arises from a pre-neoplastic adenoma generated in areas of the epithelium that are undergoing hyperproliferation. Each step correlates with the acquisition of a specific mutational event(s) (Fearon and Vogelstein, 1990). The same model is applicable to Friend erythroleukaemia (Ben-David and Bernstein, 1991). Non-neoplastic expansion of the erythroid progenitor population mediated by gp55 increases the probability of accumulating mutations that allow the eventual development of the leukaemic Friend cell, with the presence of the retroviruses increasing the mutation rate of the cells by acting as an insertional mutagen. By identifying common proviral integration sites in Friend cell lines it has been demonstrated that they almost always have deregulated expression of a member of the *ets* family of transcription factors. Furthermore, mutation of the *p53* tumour suppressor gene is found in all Friend cell lines studied to date. However, the exact role of these mutations in generating the phenotype of the leukaemic Friend cells, and in the development of other tumours, is at present unclear but is discussed at greater length below.

2.3.5: Proviral integration and Friend erythroleukaemia.

2.3.5.1: *Spi-1*.

Cloning SFFV integration sites from Friend cell lines identified a locus on chromosome 2, named *Spi-1* (*S*pecific *p*roviral *i*ntegration-*1*), that was rearranged in a very high proportion (>95%) of erythroleukaemia cell lines that had been derived from animals infected with FV-P, FV-A, Rauscher virus or SFFV alone (Moreau-Gachelin *et al*, 1988, 1989, 1990; Paul *et al*, 1989, 1991). These insertions are clustered upstream of, and in the opposite orientation to, the *Spi-1* gene. Due to the ability of the SFFV LTR to act as a transcriptional enhancer in erythroid cells (Ruscetti and Wolff, 1984), the close proximity of the LTR to the *Spi-1* gene causes

Spi-1 to become highly expressed in all these Friend cell lines. Analysis of purified erythroid progenitor cells has shown that *Spi-1* expression is normally very low during erythropoiesis, and also during the initial phases of the Friend disease, implying that deregulated *Spi-1* expression may be responsible for the leukaemic Friend cell phenotype (Tsai *et al*, 1991b; Schuetze *et al*, 1992; Galson *et al*, 1993). *Spi-1* is identical to *PU-1*, a gene cloned by virtue of the ability of its product to bind a purine rich sequence in the promoter of a major histocompatability gene and shown to be a transcription activating protein when this sequence is inserted upstream of reporter genes (Klemsz *et al*, 1990; Goebl *et al*, 1990).

Which genes Spi-1 activates in Friend cells is not known, and equally, the precise effect of deregulating *Spi-1* in these cells is a mystery. One would expect that since gp55 promotes growth factor-independent growth, *Spi-1* would cooperate in forming the leukaemic phenotype if it were to block differentiation, analogous to *v-erbA/v-erbB* cooperation seen in AEV-ES4 (section 2.2.1). Schuetze *et al* (1992) have suggested that since Spi-1 protein levels are down-regulated during the chemically-induced differentiation of Friend cells, deregulated expression must prevent differentiation. However, this is only a correlation and no functional experiments have yet been reported that test this hypothesis.

2.3.5.2: *Fli-1*.

The erythroleukaemias induced with the F-MuLV 'helper' in newborn mice, do not show activation of *Spi-1* (Moreau-Gachelin *et al*, 1989), but a different proviral integration site has been identified that is rearranged in 75%, or more, of cell lines derived from these malignancies. Insertions in this locus, termed *Fli-1* (*F*riend *l*eukaemia *i*ntegration-*1*), have not been detected in FV erythroleukaemia cell lines, or in leukaemias induced in other lineages by F-MuLV (Ben-David *et al*, 1990a; Sels *et al*, 1992). However, integration of Cas-Br-E proviruses has also been detected in this locus in a subset of neoplasms classed as non-T-, non-B-cell lymphomas that are induced on infection of newborn NIH/Swiss mice (Bergeron *et al*, 1991, 1992). A

gene has been mapped to this locus that is highly expressed in all the erythroleukaemia cell lines that contain the inserted retrovirus at the *Fli-1* locus (Ben-David *et al*, 1991). The assumption is that *Fli-1* expression is absent, or very low, in normal erythroid progenitors (although this has yet to be formally proven) and that uncontrolled expression of *Fli-1*, like *Spi-1*, is a crucial step in the progression of the leukaemia.

Fli-1 and *Spi-1* are structurally related and both members of the *ets* family of transcription factors. Both are able to activate transcription of a reporter gene linked to an appropriate recognition sequence (Klemsz *et al*, 1990; Watson *et al*, 1992; Zhang *et al*, 1993). The *GATA-1* promoter contains recognition sequences of this type, and expression of a reporter gene controlled by this promoter is up-regulated *in vivo* in the presence of Fli-1 (Watson *et al*, 1992). Two other ets-related proteins, c-ets-1 or c-ets-2, are unable to mimic this effect. This may have important implications as to why *Fli-1* is preferentially activated in some erythroleukaemias. Mechanistically, it may work in a similar fashion to that proposed for the gag-myb-ets fusion protein of ME26: up-regulation of *GATA-1* in ME26-infected cells is thought to result in the inappropriate expression of the *epoR* gene (see section 2.2.2). This suggests that *Fli-1* may potentiate proliferative stimulation by the gp55-like proteins expressed in F-MuLV-transformed erythroid cells.

The restriction of *Spi-1* activation to erythroleukaemias induced in adult mice with SFFV and *Fli-1* to F-MuLV-induced tumours in newborn mice is intriguing, but may simply be due to differences in the availability of integration sites in the developmentally distinct target cells in the two types of erythroleukaemia. Alternatively, since the Fli-1 and Spi-1 proteins exhibit considerable sequence divergence, they may regulate different sets of target genes whose expression is required in the progression of the leukaemic cells in the two systems (Ben-David and Bernstein, 1991). The structure and possible function of these and other ets-related proteins, is discussed in depth in chapter 3.

2.3.5.3: *p53* in Friend erythroleukaemia.

Mowat and his co-workers were the first to examine p53 in Friend cell lines, using antibodies to look at the protein levels. They found that approximately 30% had either no expression, or produced antigenically-related proteins that were of a considerably smaller size. Southern blot analysis of genomic DNA from these particular cell lines detected rearrangements of the *p53* gene, and complete loss of the normal allele (Mowat *et al*, 1985). These genomic abnormalities were correlated with insertion of proviruses, either SFFV or F-MuLV, or large deletions: all these events disrupted the *p53* coding sequence (Rovinski *et al*, 1987; Munroe *et al*, 1988; Hicks and Mowat, 1988; Ben-David *et al*, 1988, 1990b). Furthermore, clones in which the *p53* gene had a normal structure were found to produce proteins in which a single amino acid had been altered as a result of a point mutation. These clones were homozygous for the mutation, the wild-type allele having been lost (Munroe *et al*, 1990). Mutation of *p53* has been implicated in the process of establishing cell lines from certain cell types (Harvey and Levine, 1991). However, multiple erythroleukaemia cell clones independently derived from the same infected animal contained identical *p53* mutations, proving it had occurred *in vivo* as a component of tumour progression, and not as a consequence of culture *in vitro* (Chow *et al*, 1987). Transgenic mice constitutively expressing a mutant *p53* gene and infected with FV, develop erythroleukaemia much more rapidly than wild-type mice (Lavigueur and Bernstein, 1991). All this evidence has suggested that mutation of *p53* is an obligatory step in Friend erythroleukaemia progression, irrespective of whether these tumours are induced with FV-P, FV-A or F-MuLV (Dreyfus *et al*, 1990; Ben-David and Bernstein, 1991). Reconstituting wild-type *p53* gene expression in p53-negative Friend cells induces apoptosis, an effect reversed by erythropoietin (Ryan *et al*, 1993; Johnson *et al*, 1993). Differentiation was not observed, probably because other mutations present in these cells are preventing maturation. The role of *p53* in normal cells, and the effect its mutation has on tumour progression, is discussed in detail below (Chapter 4).

2.3.5.4: The *RNA1* gene, *c-myc* and *Pim-1*.

Another integration site has recently been identified in Friend erythroleukaemia cells. This locus, termed *Fli-2*, is rearranged in two erythroleukaemia cell lines from F-MuLV-infected animals and one from a FV-P-induced tumour, but remains intact in the majority of cell lines studied. These integrations are downstream of the heterogeneous nuclear ribonucleoprotein A1 (*RNA1*) gene that is a member of a family of genes involved in RNA splicing. However, the integrations effect expression of this gene in separate ways. In two of the cell lines, *RNA1* mRNA production appears unaltered compared with other cell lines that do not contain the rearrangement. In the other, integration in one *Fli-2* allele is accompanied by loss of the other allele and a complete absence of *RNA1* gene expression is observed. These observations are intriguing as they are the first indication that splicing factors may be targeted during tumour progression. However, the variations that integration at *Fli-2* have on *RNA1* expression imply that another gene may be the true target oncogene of these integrations (Ben-David *et al*, 1992).

A single report (Dreyfus *et al*, 1990) contained results which showed that in a small proportion of primary leukaemias induced with F-MuLV, two previously identified genes, *c-myc* (a transcription factor closely linked to a cell's proliferation status (Evan and Littlewood, 1993)) and *Pim-1* (which encodes a serine-threonine protein kinase (Selten *et al*, 1986)) had undergone rearrangement. These genes are more commonly associated with the progression of leukaemias from the T lymphoid lineage, and their involvement in erythroleukaemia is surprising. The status of the *Fli-1* gene in these cells was not reported, and it is possible that these two genes may replace the requirement for *ets* deregulation.

2.3.5.5: Over-expression of the erythropoietin receptor.

Although the epo receptor is activated by gp55 in SFFV-infected cells, Lacombe *et al* (1991) have shown that in one particular cell line, T3Cl-2, transcription of the *epoR* gene is deregulated as a consequence of insertion of the

SFFV LTR in the first exon of the gene. The coding region is not disrupted and the only effect seems to be an increase in transcription. This result implies that the proliferative advantage generated in erythroid cells by the *gp55* gene (T3Cl-2 cells express *gp55*) can be potentiated by other mutational events. It is possible that the genes more commonly activated by proviral insertions in Friend cells, such as *Spi-1* and *Fli-1*, play a similar potentiating role in erythroleukaemia development.

2.4: Summary.

The identification of genes activated or mutated by retroviral insertions in Friend erythroleukaemia has posed several questions concerning their effect on normal erythropoiesis, such as whether they block differentiation, enhance self-renewal or potentiate the growth factor-independence mediated by mutated viral envelope proteins. To gain an insight into their possible role in tumour progression it is necessary to have a full understanding of their function in normal cells and in other malignancies. Although *Fli-1* and *Spi-1* have not been extensively studied, by investigating the role of related *ets* genes it may be possible to understand why they are targeted in Friend erythroleukaemia. *p53*, on the other hand, has been the subject of intensive scrutiny over the last twelve years, but only now is the true role of this suppressor gene emerging. The following sections detail our current knowledge concerning *p53* and the *ets* genes and each concludes with a discussion of its relevance to erythroleukaemia progression.

CHAPTER 3: The *ets* genes: transcription factors and oncogenes.

3.1: Structure of the ets protein family.

Since the identification of *v-ets* in the E26 virus, over thirty *ets*-related genes have been cloned from a number of different species, all of which show significant homology over the so-called ets-domain, the part of the protein involved in sequence-

specific DNA binding (reviewed by Macleod *et al*, 1992, and Seth *et al*, 1992). The first cellular *ets* gene to be identified was *c-ets-1* (Figure 3.1): this is generally viewed as the archetypal *ets* gene and will be used in this introduction to describe properties which may be applicable to other family members. The ets-domain, which is usually found in the carboxy-terminal portion of the protein, is approximately 85 amino acids long and has no structural homology to other known DNA binding motifs, except possibly c-myb (Anton and Frampton, 1988). The c-myb protein has a DNA binding domain consisting of a triple repeat of a region containing three α-helical structures, each with a conserved, essential tryptophan residue (for review, see Graf, 1992). The ets domain of all the ets proteins, with the exception of Spi-1 and Spi-B, is like one of these repeat regions and has three conserved tryptophans, implying that it may have a triple α-helical-type structure. The two Spi proteins, which are most diverged from c-ets-1, only have two tryptophans, the other being replaced by a tyrosine (Ray *et al*, 1992). Another region of similarity has been found towards the amino-terminus of several ets proteins that exhibits weak homology to the dimerisation domain of the so-called helix-loop-helix (HLH) proteins, like c-myc and the muscle-determining factor myoD, and may be involved in mediating protein-protein interactions. In particular, in c-ets-2 this domain shows 45% identity to part of the Id protein, an HLH family member that is thought to inhibit transactivation by other HLH proteins by forming non-functional dimers (Benezra *et al*, 1990; Seth and Papas, 1990). However, no such inhibitory function has been demonstrated for the c-ets-2 protein.

The transactivation domains of the ets proteins have not been exhaustively mapped, but in c-ets-1 and c-ets-2 it appears to be split either side of the putative HLH-like dimerisation motif in a region that is the least conserved portion of the protein (Schneikert *et al*, 1992). Between the DNA binding and transactivation domains a number of the ets-related proteins also have a PEST site, an area rich in proline, glutamine, serine and threonine residues, which is thought to be a target for proteases. Cleavage at this site would create a DNA binding domain free from the regulatory constraints of the remainder of the protein that could interfere with

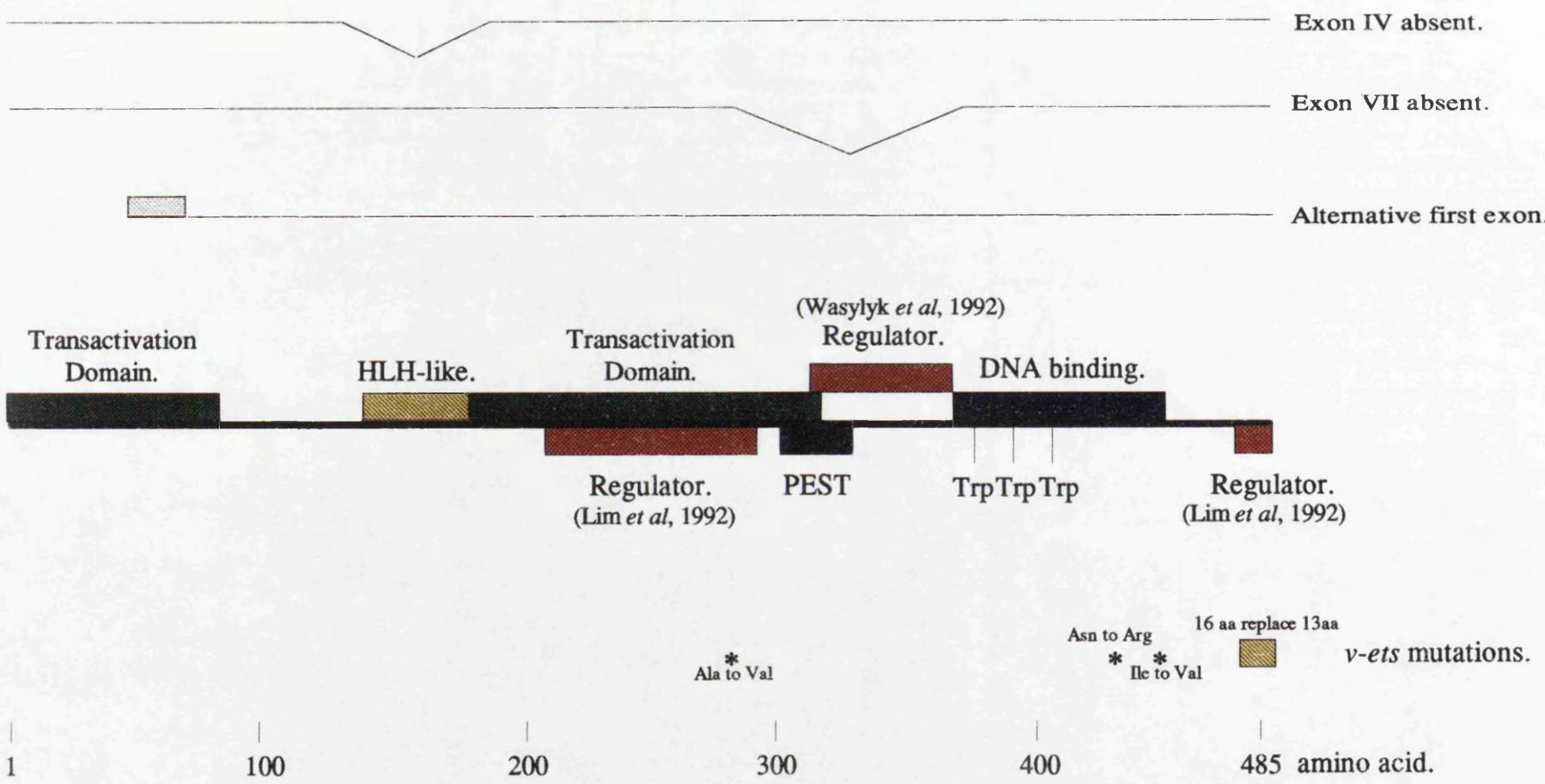


Figure 3.1: Functional domains of the c-ets-1 protein and alternatively spliced variants. (see text for further details)

transcription mediated by the full length protein. Interestingly, *ets* genes have been cloned from *Xenopus* and *Drosophila* that simply consist of an *ets* domain (Pribyl *et al*, 1988; Chen *et al*, 1988). In Spi-1, the PEST sequence is involved in protein-protein interactions with other transcription factors; dimerisation could therefore prevent the proteolytic cleavage of the ets protein (Pongubala *et al*, 1992, 1993). Several potential kinase phosphorylation sites are apparent in the ets proteins and, as is discussed below, these are probably important in regulating their function in response to extracellular signals (Watson *et al*, 1988; Koizumi *et al*, 1990).

The *ets* genes have been mapped to several chromosomes, but there are two clusters of particular interest: *Fli-1* and *c-ets-1* on human chromosome 11 (mouse 9) and *erg* and *c-ets-2* on human chromosome 21 (mouse 16) (Figure 3.2). The c-ets-1 and c-ets-2 proteins are very closely related structurally and functionally, with similar DNA binding specificities (Watson *et al*, 1988a; Wasylyk *et al*, 1990). Similarly, the Fli-1 and erg proteins are highly homologous with nearly 100% identity over their DNA binding domains, implying functional similarities (Ben-David *et al*, 1991; Reddy and Rao, 1991; Watson *et al*, 1992). These properties provide clues as to the evolution of the *ets* genes: first duplication of an ancestral *ets*-related gene may have occurred giving rise to two adjacent sister genes which subsequently diverged; later this was followed by duplication of the entire locus to another chromosome.

3.2: DNA binding: monomers and oligomers.

The similarity exhibited by ets proteins over the DNA binding domain implies that their recognition sequences are also very similar. Indeed, all the ets sites identified to date contain a central GGAA/T core, with the sequence either side involved in controlling specificity (Karim *et al*, 1990; Macleod *et al*, 1992). Many of these sites are able to bind more than one of the ets family members in gel retardation assays. For example, the PEA3 site identified in the polyoma enhancer will bind c-ets-1, c-ets-2, elk-1 and the PEA3 protein, all of which are able to activate

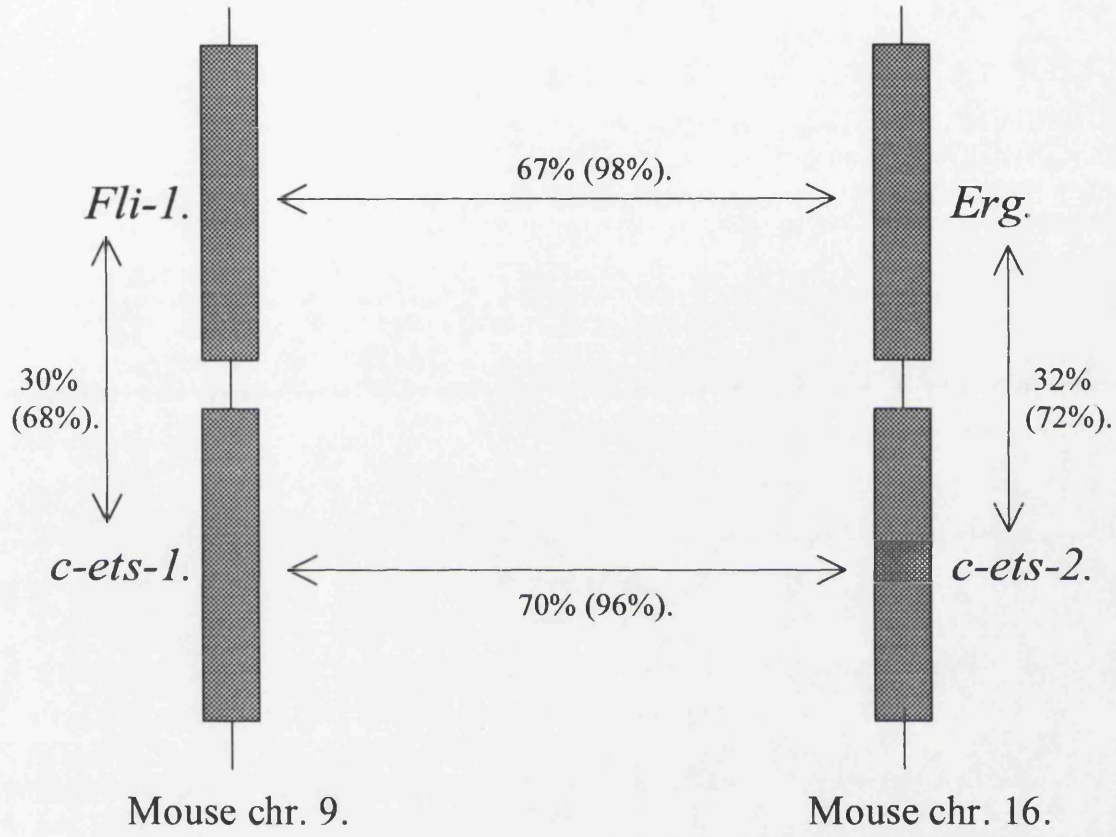


Figure 3.2: Structural similarity between four clustered, and closely related, *ets* genes.
Overall amino acid homology shown by figure without brackets.
Amino acid identity over ets domain indicated by figure in brackets.

transcription through this motif *in vivo* when it is placed upstream of a reporter gene (Wasylyk *et al*, 1990; Rao and Reddy, 1992a; Xin *et al*, 1992).

DNA binding *in vitro* occurs in the absence of any accessory proteins. *In vivo* though the situation is likely to be very different as each putative ets binding site must be considered in the context of the rest of the promoter/enhancer. An emerging rule concerning the ets proteins, some examples of which are given below, is that their DNA binding specificity and transactivation properties can be modulated by interactions with other proteins, which form an oligomeric complex over the appropriate response elements. Indeed, this was a possibility that was implied when the first ets protein, v-ets, was discovered in the E26 retrovirus (section 2.2.2), with the fusion to v-myb potentially stabilising a heterodimer formed between c-myb and a cellular ets protein.

(1) Interactions between ets-related proteins and other transcription factors were first shown by Wasylyk and his co-workers (Wasylyk *et al*, 1989, 1990) using part of the polyoma enhancer, termed the oncogene responsive domain. This region mediates transcriptional activation by serum growth factors, phorbol esters like TPA, and also by numerous non-nuclear oncogenes e.g. *v-src*, *v-raf* and *v-mos*. They showed that c-ets-1 (or c-ets-2) was induced by these stimuli to bind to the PEA3 site in the enhancer and cooperate with AP-1 fos-jun heterodimers bound at the adjacent PEA1 site. Each component activates transcription to some extent on its own, but they exhibit extensive synergism when bound at the same time. Over-expression of the *c-ets-1* gene *in vivo* results in inhibition of the transactivation of a reporter gene by AP-1 through PEA1, an observation which implies that cooperativity is mediated at the level of protein-protein interaction since the c-ets-1 protein appears to be competing out the binding of AP-1 to the PEA1 site. Furthermore, although mutations in either the PEA1 or PEA3 site diminish cooperative transactivation to some extent, this is significantly less than would have been expected considering the potent effect each mutation has on transactivation by one factor alone. In these mutant enhancers, binding of one component to its wild-type sequence stabilises the

interaction of the other factor on the mutated binding site. This cooperativity is not restricted to viral control elements since adjacent PEA3-like and AP-1 motifs have been identified in the promoters of numerous oncogene-responsive cellular promoters, including the ECM degrading enzymes stromelysin, collagenase and urokinase, the *c-fos* gene (a component of the AP-1 complex), and the *c-ets-1* and *c-ets-2* genes (explaining the autoregulation observed for these genes) (Nerlov *et al*, 1991; Wasylyk *et al*, 1991; Gutman and Wasylyk, 1991; Seth and Papas, 1990; Mavrothalassitis and Papas, 1991). The nature of the putative AP-1:ets interaction remains unresolved: there has been no demonstration of AP-1 oligomer formation with c-ets-1 or c-ets-2.

(2) A similar synergistic cooperativity in transactivation has been proposed for c-ets-2 and c-myb in the promoter of *mim-1*, a myeloid-specific gene identified due to its activation by the v-myb protein of the avian myeloblastosis virus (Ness *et al*, 1989; Dudek *et al*, 1992) . Interestingly, unlike the situation with the polyoma enhancer discussed above, c-ets-1 will not replace c-ets-2 in the control of the *mim-1* gene. Obviously, this cooperativity may have important implications as to the requirement for fusion of the *myb* and *ets* components of the E26 virus, particularly if similar cooperation is demonstrable in the promoters of genes which may be more central to transformation than *mim-1*, like *GATA-1* for instance. Thus, despite the inability to demonstrate heterodimerisation between c-myb and an ets-related protein, the E26 fusion protein may cause the constitutive expression of genes controlled cooperatively by c-myb and ets proteins.

(3) Ets proteins have also been implicated in complex formation over the serum response element (SRE). This region was first identified in the *c-fos* promoter as being essential for the rapid and transient induction of the gene by addition of serum to serum-starved cells (reviewed in Treisman, 1990). A dimer of the serum response factor (SRF) forms over this element but requires an additional factor, termed p62/TCF, before transcription is activated (Shaw *et al*, 1989). Preventing the formation of this oligomer on the SRE impairs the response of the *c-fos* gene to extracellular signals, in particular those mediated by a protein kinase-C-dependent

pathway (Graham and Gilman, 1991). Using two different experimental approaches, two proteins have been identified, SAP-1 and elk-1, that exhibit p62/TCF activity (Hipskind *et al*, 1991; Dalton and Treisman, 1992). In both these proteins, an ets-domain is found at the amino-terminus which, along with a small region adjacent to it (termed the elk-1 SRF interaction, or ESI, domain), is required for SRF:ets interaction (Janknecht and Nordheim, 1992; Rao and Reddy, 1992b; Dalton and Treisman, 1992). The elk-1 DNA binding domain alone is able to bind to the SRE, but the full length protein lacks this property due to intramolecular repression by the ESI domain. Complex formation with the SRF removes this inhibition (Rao and Reddy, 1992b). However, the full-length elk-1 protein is able to bind to other slightly different sequences, such as the PEA3 motif, without interaction with SRF (Rao and Reddy, 1992a). Thus, the elk-1 protein can interact with DNA in two ways; either it uses its intrinsic properties to bind DNA autonomously, or by interacting with other proteins, its DNA binding specificity is altered to allow other sequences to be recognised. *In vivo*, it seems likely that elk-1 could activate transcription as a monomer on one promoter type, and as part of an oligomer on another, and it is possible to envisage that subtle post-translational alterations in the DNA binding and regulatory domains of the protein could alter which particular target genes are expressed.

(4) Ets binding sites have been identified in the enhancers of numerous viruses, such as the human immunodeficiency (Leiden *et al*, 1992) and T cell leukaemia viruses (Bosselut *et al*, 1990), and F-MuLV and SFFV (section 2.3), and many of the ets proteins have been cloned as a result of binding to these sites. The enhancer of herpes simplex virus binds a complex set of host and viral proteins, including the ets protein GABP-α, which exhibits heterodimerisation properties. DNA binding by this protein requires interaction (via part of the ets domain) with repeat structures in GABP-β that are related to those seen in the cytoskeletal protein ankyrin (Thompson *et al*, 1991; LaMarco *et al*, 1991). Conversely, binding of the B-cell-specific transcription factor NF-EM5 to the immunoglobulin kappa light chain enhancer requires interaction with the phosphorylated PEST sequence of Spi-1

already bound to the enhancer (Pongubala *et al*, 1992, 1993). In this example, therefore, the ets protein is acting as a recruiting factor. Interestingly, a recent study (Hagemeier *et al*, 1993) has demonstrated that the retinoblastoma (Rb) protein is able to bind to the transactivation domain of Spi-1 *in vitro*, and probably prevents its interaction with general transcription factors on the promoter. Rb is thought to control cell cycle progression by regulating the activity of specific cellular transcription factors (reviewed in Hollingsworth *et al*, 1993): its association with Spi-1 tentatively implies that this ets protein may be part of this control pathway in some cell types.

Thus, in summary, it appears that ets proteins can bind DNA and activate transcription either as a monomer, or as a part of an oligomer. Interactions of the ets proteins with other transcription factors or accessory molecules is able to change the DNA binding specificity and/or transactivation properties of the ets proteins themselves and/or the protein(s) with which they are associating. The growing size of the ets gene family, and the complexity of protein:protein and protein:DNA interactions being elucidated for these proteins, provides an opportunity to propose numerous models to explain their role in leukaemia, but the same complexity makes a full understanding of their function extremely difficult.

3.3: Control of ets protein activity.

The activity of the ets proteins is controlled at several levels and has been studied in some detail for c-ets-1 and c-ets-2 in T-cells. Expression of all the *ets* genes is tightly controlled developmentally, temporally and spatially within a given organism. For example, *c-ets-1* is expressed at high levels in many tissues in newborn mice, but in adults the mRNA is only present in a subset of T-cells (Bhat *et al*, 1990), megakaryocytes (Lemarchandel et al, 1993), in endothelial cells involved in blood vessel formation (Wernert *et al*, 1992) and in the fibrocytic stroma surrounding invasive human carcinomas (Stehelin *et al*, 1992). The latter observation has important implications to the metastatic process, particularly in light of the known

involvement of c-ets-1 in the production of ECM-degrading enzymes (Wasylyk *et al*, 1989; Nerlov *et al*, 1991). It has been proposed that the invading carcinoma releases factors that induce *c-ets-1* expression, which in turn stimulates the production of proteases like collagenase (Stehelin *et al*, 1992). *c-ets-2*, on the other hand, is expressed in all tissues and most cell types, and in particular in cells that are proliferating rapidly (Bhat *et al*, 1987). In T cells, where both *c-ets-1* and *c-ets-2* are expressed, the genes are regulated in a reciprocal fashion on stimulation of cell proliferation (Bhat *et al*, 1990). In resting T-cells, *c-ets-2* mRNA is only present at very low levels, whilst *c-ets-1* is relatively high. Stimulation of proliferation with phorbol esters, or with antibodies to the T-cell receptor, induces a rapid production of *c-ets-2* mRNA accompanied by a down-regulation of the *c-ets-1* gene. This switch between two highly homologous proteins may maintain the expression of a certain set of c-ets-1/c-ets-2-regulated genes, whilst switching off c-ets-1-specific genes and turning on a distinct set of c-ets-2-specific genes that may be involved in proliferation induction. Indeed, like T-cells, *c-ets-2* expression is rapidly and transiently induced in macrophages and fibroblasts stimulated to proliferate with growth factors (Bhat *et al*, 1989; Boulukos *et al*, 1990). These early observations closely linked *ets* genes, and in particular *c-ets-2*, to the proliferative state of several cell types, an association that has been furthered by a number of more recent experiments discussed below (section 3.4).

Activation of T-cells also rapidly increases the phosphorylation state of the two ets proteins, an effect mediated at least in part by an as yet unidentified calcium-dependent kinase (Fujiwara *et al*, 1990; Pogonec *et al*, 1988). These modifications, mainly on serine residues, dramatically increase the stability of the c-ets-2 protein (Fujiwara *et al*, 1988), but decrease the association of c-ets-1 with chromatin and presumably restrict its ability to transactivate (Pogonec *et al*, 1989). However, phosphorylation, or some other form of modification, is probably a requirement for DNA binding by c-ets-1, since completely unphosphorylated c-ets-1 protein made *in vitro* is also unable to bind to its target sequence due to an intramolecular repression

mechanism. Lim *et al* (1992) have proposed as a result of deletion analysis of the protein, that this is mediated by an interaction between the carboxy-terminus and a repressor domain within the region identified as a transactivation domain (Figure 3.1). Interestingly, mutations present in the *v-ets* gene are in these putative regulatory domains. The thirteen carboxy-terminal amino acids of c-ets-1 are replaced by sixteen foreign amino acids (the consequence of an inversion of part of the *c-ets-1* gene (Lautenberger and Papas, 1993)), and there is a point mutation in the putative repressor domain (Figure 3.1) (Watson *et al*, 1988b). It has been demonstrated that removal of the thirteen carboxy-terminal amino acids from c-ets-1 or -2 increases their DNA binding activity (Hagman and Grosschedl, 1992; Lim *et al*, 1992). There are also two point mutations in the DNA binding domain of v-ets, the function of which remains unclear. One report suggests that these mutations actually inhibit DNA binding, although these experiments were carried out using a truncated c-ets-1 protein lacking 175 amino acids from the amino-terminus in which the true effect of these mutations may not be apparent (Leprince *et al*, 1992). In c-ets-1, this intramolecular inhibition could be disrupted by phosphorylating amino acid residues distinct from those modified on T cell activation that seem to prevent DNA binding.

To date, kinases and phosphatases that are known to directly modify ets proteins are few and far between. However, the activation of the oncogene responsive domain by non-nuclear oncogenes or phorbol esters, which is mediated by ets proteins (section 3.2), requires raf-1, a serine-threonine kinase (Bruder *et al*, 1992), although there is no evidence that demonstrates direct phosphorylation of ets proteins by raf-1. However, p62/TCF, a member of the ets family, requires phosphorylation by MAP kinase to allow transcriptional activation via the SRE (Gille *et al*, 1992), and MAP kinase itself is regulated by raf-1 via MAP kinase-kinase (Kryiakis *et al*, 1992). This phosphorylation is in the carboxy-terminal portion of the protein which functions as a regulable transcription activation domain (Marais *et al*, 1993). Post-translational modifications of p62/TCF may also alter the availability of its oligomerisation domain for interaction with SRF, which is masked in the *in vitro*-produced protein

(Janknecht and Nordheim, 1992). In a similar manner, phosphorylation of serine 148 in Spi-1, possibly by casein kinase II, is a requirement for NF-EM5 interaction and subsequent transcriptional activation by this heterodimer (Pongubala *et al*, 1993). Likewise, post-translational modifications of c-ets-1 or c-ets-2 may permit their interaction with AP-1 over the oncogene responsive domain. Theoretically, these could occur in the regulatory domains identified by Lim and his colleagues (1992). Alternatively, Wasylyk *et al* (1992) have identified a separate portion of c-ets-1 (and c-ets-2) adjacent to the DNA binding domain, that also inhibits DNA binding in the absence of phosphorylation (Figure 3.1). It is possible that this region is involved in protein-protein interactions like the equivalent portion of elk-1 (see above). Interestingly, this region, along with the DNA binding domain, are the only parts of v-ets that are indispensable to retain the leukaemogenicity of the E26 retrovirus carrying the *myb-ets* fusion gene (section 2.2.2) (T. Graf, personal communication).

Numerous alternatively-spliced *ets* transcripts have been identified that lack some of these putative regulatory domains (Figure 3.1). For example, the regulatory region identified by Wasylyk *et al* (1992) is absent from an alternatively-spliced human *c-ets-1* lacking exon VII (Koizumi *et al*, 1990). A similar mouse variant is unaffected in its ability to bind to DNA on T-cell activation unlike the full-length protein (Pogonec *et al*, 1990). Using PCR, Jorcyk *et al* (1991) identified *c-ets-1* transcripts that lacked exon IV and others lacking IV and VII. Exon IV contains the helix-loop-helix homology domain identified by Seth and Papas (1990). In chickens, alternative-splicing of the *c-ets-1* mRNA creates proteins with different amino-termini, with c-ets-1(p54) missing a transactivation domain present in c-ets-1(p68) (Leprince *et al*, 1988). Interestingly, these two variants are expressed in different cell types with p54 preferentially expressed in lymphoid cells in the thymus and spleen, whilst p68 appears to be present in non-lymphoid cells, probably endothelial cells, in the spleen only (Leprince *et al*, 1988, 1990). The exact role of these different proteins is unknown, but it seems likely that their transactivation properties will vary, and that they will be subject to distinct regulatory processes.

Thus, activity of ets proteins is regulated at the level of gene transcription, by alternative splicing and intramolecular repression, and also by post-translational modifications. Much more work is still required to determine how these regulatory steps are controlled by extracellular signals, and also if these mechanisms are common to all ets proteins. This seems likely as many of the other ets-related proteins are highly tissue specific which can be produced from alternatively spliced transcripts and are phosphorylation substrates for cellular kinases (Ben-David *et al*, 1991; Ray *et al*, 1992; Watson *et al*, 1992; Xin *et al*, 1992; Reddy *et al*, 1987; Gille *et al*, 1992; Galson *et al*, 1993).

3.4: Intracellular signalling and the ets proteins.

Control of cell proliferation and maturation is mediated by extracellular molecules binding to receptors on the target cell to elicit a complex intracellular response that eventually culminates in alterations in the genes being expressed in that cell. Most cellular proto-oncogenes are components of these signalling pathways, and the genetic alterations found in transforming oncogenes result in signalling proceeding in the absence of the appropriate stimulus. The involvement of ets proteins in the oncogene responsive domains of cellular and viral promoters, and in the serum response process, is a strong indication that they are a nuclear component of mitogenic pathways. Many other genes that contain ets binding sites are rapidly induced with extracellular signals: for example, a large number of cytokine genes contain ets binding sites (Klemsz *et al*, 1990; Seth *et al*, 1992; Thompson *et al*, 1992). The rapid changes in the phosphorylation state of the ets proteins on cell stimulation, some of which is mediated by kinases known to be crucial in mitogenic cell signalling like MAP kinase, and the *in vitro* interaction of Spi-1 with the Rb protein, is further evidence that these proteins play a role in mediating a cell's response to proliferation-inducing stimuli.

There is now emerging some direct functional evidence for a role in proliferation induction. First, infection of chicken erythroid cells with *v-ets* results in

growth factor-independent growth of cells still able to differentiate, and will cooperate with *v-erbA*, a differentiation-blocking oncogene (section 2.2.1), to generate erythroleukaemias (Metz and Graf, 1991b, 1992). Thus, *v-ets* mimics the *in vitro* transformation and cooperative leukaemogenic effects observed with the mutated EGF receptor, *v-erbB*, and other kinase-type oncogenes e.g. *v-raf*, *v-src* (Klinken, 1988). Second, over-expression of *c-ets-1* or *c-ets-2* in fibroblasts causes transformation, as defined by increased proliferation and an induction of tumorigenicity (Seth *et al*, 1989; Seth and Papas, 1990). Third, Langer *et al* (1992) have recently shown that over-expressing a dominant-negative *c-ets-2* mutant containing only the DNA binding domain of the protein, prevents mitogenic stimulation of cells by colony-stimulating factor-1 (CSF-1). Induction of *c-ets-2*, *c-jun* and *c-fos* by CSF-1, which is seen in the absence of the c-ets-2 mutant, still occurred, but *c-myc* expression was severely reduced. Furthermore, CSF-1 signalling was restored in NIH 3T3 cells expressing a mutant CSF-1 receptor if a full-length *c-ets-2* gene was artificially over-expressed. These same cells can be rescued for CSF-1-responsiveness if *c-myc* is over-expressed (Roussel *et al*, 1991): a similar phenomenon is observed with *c-myc* expression constructs introduced into cells containing the wild-type receptor and the dominant-negative c-ets-2 mutant. In short, these results suggest that c-ets-2 plays a mediating role in the transduction of CSF-1 signals to *c-myc* expression. Indeed, preliminary evidence quoted by Langer and his colleagues (1992), reports the identification of a number of ets-binding sites in the promoter of the *c-myc* gene. These observations, in conjunction with the work on the serum response element, demonstrate that ets proteins are involved in regulating the expression of several immediate-early genes that are rapidly induced by proliferative signals, and thought to facilitate a cell's progression through the cell cycle (for reviews see Gillespie, 1991 (fos/jun) and Evan and Littlewood, 1993 (myc)).

It is possible that proliferation could also be induced indirectly by autocrine production of growth factors, since several cytokine promoters contain putative ets-binding sites (Seth *et al*, 1992; Klemsz *et al*, 1990). For example, mitogenic

activation of T-cells is potentiated by the autocrine production of interleukin-2 and its receptor (reviewed in Smith, 1988). *IL-2* gene expression requires binding elf-1, an ets-related protein, to a site within its promoter (Thompson *et al*, 1992). Alternatively, growth factor receptor expression may be controlled by ets-related proteins. For example, as discussed in section 2.2.2, in cells transformed by the ME26 virus (the mouse homologue of E26), the myb-ets fusion protein induces GATA-1 expression which in turn results in the inappropriate expression of the epo receptor in cells not usually epo-responsive. Similarly, the Fli-1 protein, unlike c-ets-1 and -2, can induce the expression of a reporter gene linked to the *GATA-1* promoter which may explain the selective activation of *Fli-1* in F-MuLV-induced erythroleukaemias (Watson *et al*, 1992). However, it has yet to be demonstrated whether *GATA-1* or *epoR* expression is increased in F-MuLV-transformed erythroid cells, and what implications this result has, if any, on their proliferation. Finally, *in vivo*, the production of ECM-degrading enzymes, which appear to be regulated by ets proteins, may also play a role in the proliferative response by destroying restrictive signals present in the microenvironment. The microenvironment of the haemopoietic organs is crucial to the regulation of blood cell production (chapter 1), and any disruption may affect the progression of developing leukaemic cells.

There is also some suggestive evidence that ets proteins may be involved in the control of differentiation. It has been proposed that activation of *Spi-1* in FV-induced erythroleukaemia cells, already in a highly proliferative state due to the gp55-epo-R interaction, is involved in blocking differentiation (Schuetze *et al*, 1992). No functional evidence was presented in this report, and the conclusion was based on the observation that Spi-1 protein levels dropped significantly when Friend cells were forced to differentiate in response to chemical inducers. More compelling evidence comes from the observation that the multipotent cells transformed with a mutant E26 virus containing a temperature-sensitive mutation in the v-ets component of the fusion protein, undergo terminal erythroid differentiation at the non-permissive temperature (Golay *et al*, 1988). However, it is a distinct possibility that differentiation may be a

result of decreased cell division, and indeed, it is feasible that an oncogene which increases the proliferative potential or life-span of a progenitor cell may block differentiation simply as a consequence of that increased viability. Moreover, many of the chemicals used to artificially induce the differentiation of leukaemic cells *in vitro*, act by blocking DNA replication (Terada *et al*, 1977), presenting differentiation as a default pathway when a cell's proliferative potential is exhausted or inhibited. If this is true, then ets proteins do have the potential to play a dual role in controlling proliferation and maturation of erythroid cells, simply by increasing proliferation.

With respect to a more direct effect on differentiation, it is worth remembering that many of the genes known to be regulated by members of the ets family, code for lineage-restricted proteins; for example certain cytokines, like IL-2, the immunoglobulin kappa light chain, mim-1, the ECM-degrading enzymes and several cell adhesion molecules (Pahl *et al*, 1993; Lemarchandel *et al*, 1993). One could envisage a mechanism whereby deregulated expression of an *ets* gene could disrupt the expression of these proteins, and thus prevent normal maturation, perhaps by a dominant-negative mechanism akin to v-erbA in AEV (section 2.2.1). The observation that the Fli-1 protein can induce the expression of a reporter gene linked to the *GATA-1* promoter, also has possible implications in the differentiation block observed in F-MuLV-induced erythroleukaemias (Watson *et al*, 1992), bearing in mind that GATA-1 is central to the expression of many erythroid-specific genes. Furthermore, several recent reports have identified cooperative transactivation effects between GATA-1 and ets-related transcription factors in the regulation of certain lineage-restricted haemopoietic genes. For example, Spi-1 is able to bind to a sequence, adjacent to a GATA-1 site, in the regulatory regions of the β-globin gene (Galson *et al*, 1993), whilst erythroid expression of glutathione peroxidase seems to require GATA-1 and ets proteins binding to an enhancer 3' to the gene (O'Prey *et al*, 1993). Similarly, in megakaryocytes, GATA-1 and ets-related proteins regulate the expression of a lineage-restricted integrin (Lemarchandel *et al*, 1993).

3.5: *ets* genes and human tumours.

The activation of *Spi-1* and *Fli-1* in Friend erythroleukaemias in mice, and the role *v-ets* plays in the leukaemogenicity of the avian E26 virus, is very strong evidence that these genes are involved in tumour progression. Furthermore, in rare rat thymic lymphomas induced by the Moloney MuLV, *c-ets-1* is activated as a result of viral insertion (Bear *et al*, 1989; Seth *et al*, 1992). Erythroleukaemias are relatively rare in humans, representing as few as 5% of leukaemias, and have not been extensively analysed cytogenetically or at a molecular level to identify genes involved in their evolution, although Klemsz *et al* (1993) have reported that a subset of these malignancies express the *Fli-1* gene.

Translocation breakpoints in other leukaemias are often closely associated with regions of chromosomes where *ets* genes are located (reviewed in Papas *et al*, 1990). For example, the *c-ets-1/Fli-1* region on chromosome 11q23 is a constitutive and heritable fragile site (Yunis and Soreng, 1984), and a frequent breakpoint in leukaemias and lymphomas (Diaz *et al*, 1986; Rovigatti *et al*, 1986), whilst the *c-ets-2/erg* site on chromosome 21q22 is involved in a translocation with chromosome 8 in acute myelogenous leukaemia (Sacchi *et al*, 1986; Rao *et al*, 1988). Interestingly, *erg* and *c-ets-2* are located in the minimal trisomic region in Down's syndrome sufferers: coincidentally, these individuals have an increased susceptibility to developing leukaemias, in which *erg* and/or *c-ets-2* gene dosage may play a part (Papas *et al*, 1990). However, in all these examples, the role of the *ets* genes has yet to be directly proven. Indeed, recent work cloning breakpoints from these sites, has identified novel genes at 11q23 and 21q22 that are disrupted by the translocations present in these leukaemias (Tkachuk *et al*, 1992; Gu *et al*, 1992; Djabali *et al*, 1992; Ziemin van der Poel *et al*, 1992; Miyoshi *et al*, 1991; Chen *et al*, 1993; McCabe *et al*, 1993). In both cases, there is no homology to the *ets* gene family, yet this does not necessarily exclude a role for the closely-linked *ets* genes in tumour progression. For example, in chronic myeloid leukaemia there is no correlation between a (3;21) translocation and *c-ets-2* expression in chronic-phase patients, but all acute-phase patients tested

express *c-ets-2* (Lafage-Pochitaloff *et al*, 1992): it is possible that translocations have secondary effects on adjacent genes that are selected for during tumour development.

Only one example exists that unequivocally shows *ets* gene involvement in human tumorigenesis. Cytogenetic analysis of Ewing's sarcoma and related neoplasms derived from primitive neuroectodermal tissue has demonstrated that these tumours always have translocations between specific parts of chromosomes 11 and 22, a property that has been used for diagnostic purposes (reviewed in Granowetter, 1992). This abnormality fuses *Fli-1* to a putative RNA binding protein termed EWS (Delattre *et al*, 1992). The expressed fusion protein contains the DNA binding domain of Fli-1 linked to the amino-terminal portion of EWS, a region that contains considerable homology to a subunit of the RNA polymerase II complex. The effects of this protein are two-fold. First, it results in the ectopic expression of the Fli-1 DNA binding region that may disrupt DNA binding and transactivation by other *ets* genes and activate other specific target genes itself. Second, the EWS component may mimic or interfere with the normal action of RNA polymerase II, the Fli-1 portion bringing it in close proximity to gene promoters and enhancers.

3.6: Summary.

The ets family is an ever-increasing group of closely-related transcription factors which bind to similar recognition sequences, either as monomers or as part of an oligomeric complex. Several studies have demonstrated that the DNA binding activity and sequence specificity of many of these proteins is dependent on post-translational modifications and/or oligomer formation, but the role of these proteins *in vivo* remains largely unresolved. However, an emerging theme is that they are involved in controlling the expression of other genes known to be induced during proliferation induction, or during the process of lineage-specific differentiation. In each case, these genes are controllable by extracellular stimuli which presumably alter the activity of a specific ets protein, via a signal transduction cascade. Considering these observations, it is not surprising that a number of the *ets* genes have oncogenic

potential and are involved in the progression of certain malignancies, such as the deregulated expression of *Fli-1* and *Spi-1* in Friend erythroleukaemias. These genetic alterations could permit the constitutive activation of certain signal transduction pathways in the absence of the appropriate stimuli, or interfere with the action of other closely-related ets proteins. The development of an erythroid cell system which shows variation with respect to differentiation and growth factor or stromal cell signalling, should allow a detailed appraisal of these possibilities, first by looking for correlations between expression and phenotype, and then by testing the significance of these correlations by ectopic *ets* gene expression.

CHAPTER 4: p53.

4.1: Historical perspective.

p53, a nuclear phosphoprotein, was originally identified by its ability to form a high affinity complex with the large T antigen of simian virus 40 (SV40) (Lane and Crawford, 1979; Linzer and Levine, 1979). Tumour-derived or transformed cells were shown to produce abnormally large amounts of the p53 protein, which was barely detectable in normal cells. Furthermore, cDNA and genomic clones were isolated from libraries made from normal and malignant cell DNA, that could immortalise cells in culture and cooperate with activated *ras* to transform primary rat embryo fibroblasts (REFs) (Eliyahu *et al*, 1984; Jenkins *et al*, 1984; Parada *et al*, 1984). These results implied that *p53* was a classical dominant oncogene, activated by over-expression. However, this was challenged by several observations. First, Jenkins and his colleagues demonstrated that the inability of a particular *p53* construct to transform cells could be overcome by introducing small deletions into the *p53* coding sequence (Jenkins *et al*, 1985). Second, there was accumulating evidence from Friend erythroleukaemia cell lines that gross disruptions and deletions in the *p53* gene appeared to be associated with tumour progression (section 2.3.5.3). Third,

Rovinski and Benchimol (1988) showed that a presumed wild-type (wt) *p53* genomic clone, controlled by its own promoter, was as efficient at transforming REFs as grossly mutant clones isolated from the Friend cell lines. At the time, this suggested that wt*p53* could be oncogenic as a result of very subtle changes in expression and/or gene dosage. Finally, it was observed that there were small sequence differences between cDNA clones that were transforming and those that were not (Finlay *et al*, 1988). Furthermore, comparison of *p53* sequences from different species had identified five evolutionarily conserved domains (reviewed in Soussi, 1990) into which these differences mapped, implying that they may alter the function of the protein. Subsequently, it was shown that all the *p53* clones capable of cooperating with activated *ras* in transformation, including the 'wt' gene used by Rovinski and Benchimol (1988), were in fact mutants (Eliyahu *et al*, 1988; Hinds *et al*, 1989). In view of the gross alterations seen in the Friend cell lines, it was assumed that mutation of *p53* was a loss-of-function alteration and that their ability to transform was a consequence of a dominant-negative effect over the wt protein in these cells (discussed in length in section 4.4 below). This was supported by a crucial set of experiments performed by Finlay and her co-workers (Finlay *et al*, 1989), in which it was demonstrated that co-transfection of wt*p53* significantly reduced REF transformation by mutant *p53* and activated *ras*. Furthermore, wt*p53* had a similar effect on transformation induced by a combination of activated *ras* and the adenovirus *E1a* gene; any transformed clones that did develop lacked wt*p53* expression. Thus, *p53* was finally classified as a tumour suppressor gene.

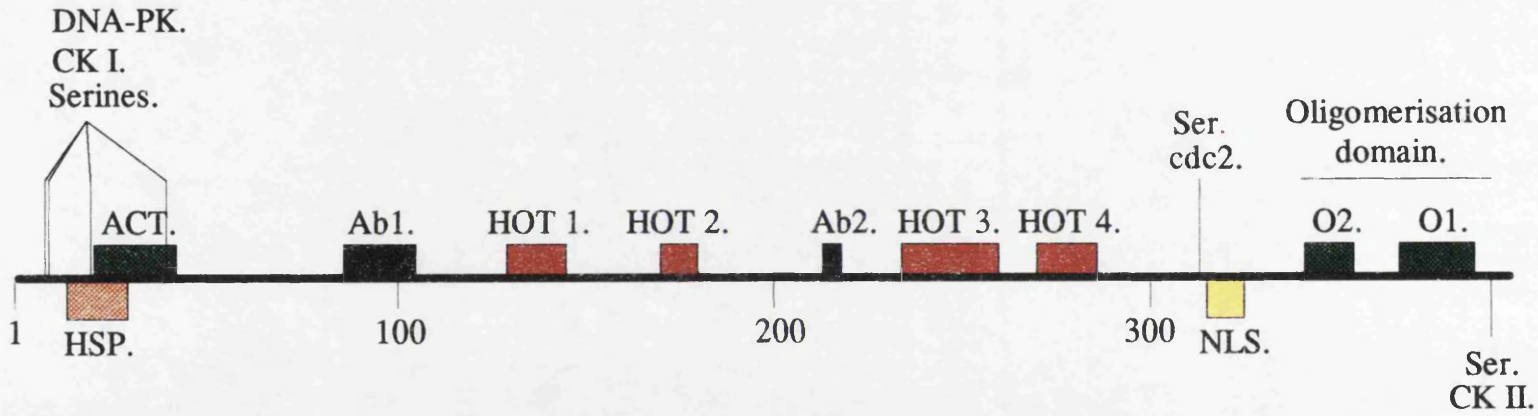
4.2: Mutation of *p53* during tumorigenesis: loss of growth suppression.

Mutation in one copy of the *p53* gene, usually accompanied by loss of the other wt allele, is the commonest genetic alteration detected to date in human tumours, and is involved in tumour progression in numerous animal model systems. This indicates the importance of this gene in controlling normal cell growth and justifies the extensive studies that have been performed concerning its properties. As

discussed above, *p53* mutation appears to be an obligate step in the generation of leukaemic cells in Friend disease, but it has also been shown to be involved in other animal model systems. For example, formation of chemically-induced carcinomas in mouse skin often requires mutation of the *p53* gene (Burns *et al*, 1991). In humans, it was demonstrated that allelic deletions of the short arm of chromosome 17, in particular region 17q12 to 17q13.3, are associated with tumours derived from various cell types (Baker *et al*, 1989). This region contains the *p53* gene, and subsequent sequencing of the remaining allele, first from colorectal carcinomas, but later from a wide variety of tumour types, revealed that it had become mutated (Baker *et al*, 1989; Nigro *et al*, 1989; reviewed in Hollstein *et al*, 1991). These alterations usually take the form of a base change, either transition or transversion, that changes a single amino acid in the mature protein. Occasionally, small deletions or insertions are found that usually shift the reading frame resulting in production of a truncated protein. Also, a number of splicing mutants have been characterised that generate transcripts that lack exons and/or contain intronic sequences with the result that the encoded proteins are truncated by the introduction of a stop codon. A comprehensive review of *p53* mutations has been made by Hollstein *et al*, 1991, and Caron de Fromentel and Soussi, 1992.

Unlike the activating mutations found in *ras* which are targeted to a few codons (reviewed in Bos, 1988), *p53* mutations are widely spread throughout the gene. However, approximately 98% of the base substitutions are found in the central portion of the gene coded by exons 5 to 8, clustered in the evolutionarily conserved domains (labelled 'HOT' in Figure 4.1) (Soussi, 1990). Nearly 90% of these alter an amino acid that is invariant between species as widely diverged as trout and man, implying a functional importance to these changes. Interestingly, the type of mutation and its position varies between tumours of different origin. This has implied the direct involvement of environmental carcinogens in mutations found in lung, liver and skin tumours. For example, individuals exposed to aflatoxin B1 and the hepatitis B virus have mainly G to T transversions clustered in codon 249 (Bressac *et al*, 1991;

Figure 4.1: Domain structure of the p53 protein.



KEY.

HSP: Binding site for heat shock protein.

ACT: Minimal transactivation domain.

CKI: Casein kinase I-like phosphorylation site.

Ser. CKII: Phosphorylation site for casein kinase II.

DNA-PK: double-strand DNA-dependent protein kinase phosphorylation site.

Ab1: Epitope for PAb246.

HOT: Mutational 'hot-spots'.

Ab2: Epitope for PAb240.

Ser. cdc2: Phosphorylation site for cdc2 kinase.

NLS: Nuclear localisation signal.

O1 and O2: Oligomerisation domain.

For further description and appropriate references, see text.

Hsu *et al*, 1991); G to T mutations are also induced with benzo[*a*]pyrene, a major constituent of tobacco smoke (Ruggeri *et al*, 1993). Similarly, CC to TT double base changes are most common in skin cancer; these types of alterations are induced by UV light (Brash *et al*, 1991; Zeigler *et al*, 1993). However, mutations in other tumour types, especially in colorectal carcinoma, are often G:C to A:T transitions occurring at CpG dinucleotides. These sites become methylated on the cytosine residue: as a consequence, they can undergo spontaneous or enzymatic deamination to a thymidine residue generating a transition mutation (Sved and Bird, 1990; Shen *et al*, 1992). Thus, CpGs act as endogenous mutagens, and indeed, it is thought that approximately 35% of human genetic disorders are the result of mutations at these sites, despite being under-represented by a factor of five in the genome (Rideout *et al*, 1990).

Germ-line mutations in the *p53* gene, half of which involve CpG dinucleotides, appear to be responsible for the Li-Fraumeni syndrome (LFS), characterised by the early development of tumours (median age of presentation is approximately 30 years of age) at different sites within the body. Breast cancer, soft tissue sarcomas and brain tumours are particularly common (Malkin *et al*, 1990; Srivastava *et al*, 1990, 1992). LFS individuals are heterozygous for these mutations still retaining a copy of the wt gene, although losing this allele during tumour formation. Thus, development of LFS-associated tumours requires only one mutational event to remove the suppressive effect of the p53 protein, whilst two mutations are necessary in sporadic tumour progression. This disease has now been mimicked to some extent in genetically-engineered mice that only contain one copy of the *p53* gene, the other being deleted by homologous recombination (Donehower *et al*, 1992). These animals have an increased susceptibility to tumour formation, which can be increased dramatically by deleting the other allele to create a *p53*-null animal. These null mice appear to be developmentally normal but tumours form within three months of birth, usually in the lymphoid tissues. Prior to these results, *p53* mutation had been implicated as a late event in tumour progression, for example in the adenoma to carcinoma step in colon cancer (Baker *et al*, 1990a) and in the blast crisis

of chronic myelogenous leukaemia (Ahuja *et al*, 1989; Feinstein *et al*, 1991). LFS and the *p53*-null mice suggest that it is the accumulation of mutations, not necessarily the order in which they occur, that is important in tumorigenesis. Indeed, as discussed in detail below, there is increasing evidence to suggest that mutation of *p53* may actually facilitate the accumulation of these mutations.

By transfection of wt*p53* under the control of a viral LTR, it has been demonstrated that the growth and tumorigenicity of several tumour cell types is inhibited by the re-expression of the wt protein (Baker *et al*, 1990b; Chen *et al*, 1990; Diller *et al*, 1990; Casey *et al*, 1991). The identification of a temperature-sensitive (ts) *p53* mutant allowed Michalovitz and his colleagues (1990) to confirm these results elegantly by simply altering the incubation temperature of tumour cells transfected with the mutant. At 37.5°C, the cells grow normally, whereas on shifting to 32.5°C causes the protein to act as a suppressor and inhibit cell growth. Inhibition appears to be the result of a block in the cell cycle in late G1, just prior to DNA replication (Lin *et al*, 1992). In two cell lines tested, derived from a myeloid leukaemia and a colon carcinoma, wt*p53* induced apoptosis, implying that in some cell lines at least, *p53* has more than a cytostatic role (Yonish-Rouach *et al*, 1991; Shaw *et al*, 1992). Furthermore, in a separate experiment, Feinstein and her co-workers (1992) reported that wt*p53* could induce the differentiation of K562 along the erythroid lineage, whilst Shaulsky *et al* (1991a) detected mature B-cell formation on transfection of the normal gene into a B-lymphoma cell line. This may simply be because arrest in the cell cycle causes differentiation, rather than cell death, in certain cell types. Alternatively, it is possible that these results actually reflect an induction of apoptosis but that cytokines present in the cultures encourage maturation rather than cell death once cells stop proliferating. Indeed, Yonish-Rouach and colleagues (1991) could inhibit *p53*-induced apoptosis by treatment with interleukin-6 which induced the cells to differentiate along the monocytic lineage instead. An important aspect of haemopoietic cytokine biology is their ability to suppress apoptosis (see Erythropoietin section in chapter 1 and Williams *et al*, 1990b). Furthermore, as

discussed in section 2.3.5.3, reconstitution of wtp53 in Friend erythroleukaemia cells lacking any p53 proteins induces apoptosis, an effect that can be reversed to some extent by the addition of erythropoietin (Ryan *et al*, 1993; Johnson *et al*, 1993). Differentiation is not induced in this case, probably because of other mutations present in the cells that can not be overridden by the wtp53 protein or the epo.

4.3: p53 and viral oncoproteins.

SV40, some adenoviruses and human papilloma virus (HPV) type 16 and 18 are capable of initiating tumours in animals and transforming cells *in vitro*. Certain viral oncoproteins are known to be both necessary and sufficient for this: these are the SV40 large T and small t antigens, E1a, E1b-55kDa and E1b-19kDa from the adenoviruses and HPV E6 and E7. These proteins exhibit a wide variety of effects within a host cell, such as altering transcription or affecting mRNA stability and processing; however, probably the most important functions a number of these proteins perform is to inactivate the p53 and Rb tumour suppressor proteins (reviewed in Lane, 1989). The SV40 large T antigen, adenovirus E1b-55kDa and HPV E6 are all able to bind to p53 (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow *et al*, 1982; Werness *et al*, 1990); more recently the x antigen of the hepatitis B virus has been added to this list (Feitelson *et al*, 1993). Tumours generated in transgenic mice expressing the large T antigen, and human cervical cancers which show HPV involvement, do not have mutations in their *p53* genes (Moore *et al*, 1992; Crook *et al*, 1992), presumably because these viral proteins inactivate wt function. On the other hand, non-HPV-associated cervical tumours often do have mutant *p53* genes.

The E6 protein prevents tumour suppression by p53 by increasing its rate of degradation, virtually removing it all from HPV-infected cells (Scheffner *et al*, 1990). However, large T and E1b-55kDa stabilise p53 and probably inhibit its function by sequestration into inactive complexes, similar to the way mutant p53 is thought to exert its dominant-negative effect (section 4.4). Indeed, large T mutants that are unable to bind p53 are transformation-incompetent (Peden *et al*, 1989) although other

work has implied that SV40 can overcome the antiproliferative effects of wtp53 in the absence of detectable large T-p53 complex formation (Michalovitz *et al*, 1991). Furthermore, free p53 in SV40-transformed cells is as stable as p53 bound to the large T antigen, results that have led to the proposal that p53 inactivation may involve a covalent modification that is either prevented or induced by large T (Deppert and Haug, 1986). Nevertheless, the targeting of p53 by these viruses reiterates the importance of p53 in preventing tumour formation, and shows that mutation of the gene is not the sole mechanism of p53 inactivation.

4.4: Properties of mutant p53: protein structure, and dominance over wild-type.

Early in the study of p53 it was noticed that mutant protein present in tumour cells is highly stable, possibly a consequence of interaction with heat shock proteins mediated by a domain between amino acids 13 and 29 at the very amino-terminal end of the p53 protein (Oren *et al*, 1981; Finlay *et al*, 1988; Lam and Calderwood, 1992). This association may result from the inability of the mutant to fold into the wt conformation, thus revealing the amino-terminus. Indeed, Gannon and his co-workers (1990) generated an antibody (PAb240) that specifically recognises many of the mutant p53 proteins, but will not bind to the wt form. These p53 mutations have, therefore, altered not just the sequence of the protein but also its conformation Irrespective of the position of the mutation, the epitope for this antibody, which is located in the centre of the protein between conserved regions 3 and 4 (Stephen and Lane, 1992; Figure 4.1), becomes exposed.

Unlike the retinoblastoma protein that becomes non-functional on mutation, the ability of mutant *p53* genes to transform primary REFs in combination with activated *ras* (section 4.1), suggests that it has obtained a new function in addition to losing its ability to act as tumour suppressor. Also, transgenic mice that contain a mutant allele and two normal alleles have an increased susceptibility to tumour formation compared to normal mice, despite still requiring two mutational events to remove all wt*p53* (Lavigueur *et al*, 1989). In fact, these animals get tumours at about

the same age as heterozygous *p53*-null mice that only have one wt allele (Donehower *et al*, 1992). These results suggest that some *p53* mutations act as dominant-negative inhibitors of wtp53 function.

However, other mutations, including LFS mutations, do not confer this property; whilst these genes are unable to inhibit REF transformation, they are also only weakly transforming, in effect behaving like recessive-negative mutants (Halevy *et al*, 1990; Hinds *et al*, 1990; Malkin *et al*, 1990). The *p53* truncation mutants seen in LFS and in sporadic tumours (see section 4.2 and Sameshima *et al*, 1992), are also thought to be of this type. It is possible that this is necessary to permit the survival of LFS individuals to adulthood, to allow the subsequent transmission of the mutant allele. Interestingly, Prosser *et al* (1992) have reported a *p53* mutation in a family prone to developing tumours much later than LFS family members, suggesting that this mutation may be an even 'weaker' dominant-negative allele than LFS mutations. However, it can not be excluded that the genetic background and lifestyle of this family may also prevent the early appearance of tumours.

p53 dominant-negative mutant proteins are believed to work by a process of sequestration of the wt protein into non-functional mutant-wt oligomers. Oligomerisation of wtp53, and in particular tetramer formation, has been demonstrated *in vivo* and is known to be mediated by two contiguous motifs in the carboxy-terminus (Figure 4.1) (Kraiss *et al*, 1988; Milner and Medcalf, 1991; Stenger *et al*, 1992; Sturzbecher *et al*, 1992; Friedman *et al*, 1993). However, the formation of mutant-wt complexes has been difficult to mimic *in vitro*. Using the ts *p53* dominant-negative mutant (section 4.2), it has been possible to show that, when in the mutant conformation, mixed complexes of mutant and endogenous wtp53 protein form in the cytoplasm; however, these are lost when the ts mutant acquires its wt properties (Martinez *et al*, 1991). Interestingly, protein synthesis is necessary for this sequestration (Gannon and Lane, 1991), and recent work has indicated that it may be synthesis of p53 itself that is required, since only when mutant and wt*p53* are co-translated can they form oligomers *in vitro* in which the wtp53 is driven into the

mutant PAb 240-positive conformation (Milner and Medcalf, 1991; Milner *et al*, 1991). Truncated p53 proteins lacking the carboxy-terminal oligomerisation domain are unable to complex with, and alter the conformation of, the wtp53 protein, and are thus, unable to exert a dominant-negative effect. Intriguingly, p53 miniproteins, essentially containing only the oligomerisation domain, are highly effective dominant-negative transforming proteins, presumably by a mechanism of sequestration (Shaulian *et al*, 1992).

However, it seems unlikely that any of the mutant p53 proteins are totally dominant over the wt protein, since tumour cells expressing a mutant *p53* gene invariably lose the wt allele by another mutational event. It is possible that in a developing tumour cell containing both wtp53 and dominant-negative mutant p53, some wt protein is not sequestered and mutant dominance is only partial, but nevertheless sufficient to give the cell a growth advantage over its neighbours. Complete loss of the wt by a second mutation could then also be seen as a progressive step to tumour formation, explaining the reduction to homo- or hemizygousity that is seen in most tumours.

There is also evidence suggesting that mutant p53 may gain an additional oncogenic function since the tumorigenicity and plating efficiency in semi-solid media of *p53*-negative cell lines is increased by expressing a mutant *p53* gene (Wolf *et al*, 1984; Shaulsky *et al*, 1991b; Dittmer *et al*, 1993).

4.5: Properties of wtp53, a transcription factor.

Until very recently the role played by p53 in normal cells remained somewhat mysterious. However, there is now accumulating evidence that wtp53 is a transcription factor (reviewed in Vogelstein and Kinzler, 1992). First, it was noted that a fusion protein containing p53 linked by its carboxy- or amino-terminus to the GAL4 DNA binding domain could activate transcription from the GAL4 operon (Fields and Jang, 1990; Raycroft *et al*, 1990; O'Rourke *et al*, 1990). More detailed work has narrowed this effect down to the region between amino acids 20 and 42,

many of which are acidic, which is now considered to be the minimal transactivation domain (Figure 4.1) (Unger *et al*, 1992; Miller *et al*, 1992). Second, by screening genomic DNA fragments, several groups have identified DNA sequences that specifically bind to wtp53 *in vitro*: these have a consensus that requires two copies of the 10bp motif 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3' separated by up to 13bp of random sequence (Kern *et al*, 1991b; El-Deiry *et al*, 1992; Funk *et al*, 1992; Foord *et al*, 1993). The requirement for two copies of a symmetrical sequence for the binding of wtp53 to a specific DNA sequence *in vitro*, implies that wtp53 protein may bind as a tetramer. However, *in vivo* it is possible that one copy of the motif is sufficient for p53 binding, if adjacent factors cooperate to stabilise the p53-DNA complex.

This evidence alone does not prove that p53 is a transcription factor. However, it is supported by transient co-transfection experiments in which transfection of p53-negative cell lines with a reporter gene linked to a p53 binding site, along with a *p53* expression vector, causes up-regulation of reporter gene transcription (Kern *et al*, 1992; Funk *et al*, 1992). Although this could be mediated by other cellular proteins controlled by p53, Vogelstein and Kinzler (1992) have argued that p53 is having a direct effect on the promoter for the following reasons. First, the experiments are repeatable in yeast (Scharer and Iggo, 1992; Kern *et al*, 1992): if other cellular proteins mediated the transcription there would have to be remarkable conservation in evolution between yeast and mammals, even though there is no known *p53* homologue in yeast. Second, Farmer *et al* (1992) have been able to show transcriptional activation by p53 *in vitro* using purified wtp53 and similar reporter constructs. Third, by using a number of different reporter plasmids, Kern and his colleagues (1992) were able to correlate the strength of p53 binding to its recognition site to the extent of transactivation.

4.6: Target genes and DNA replication.

Co-transfection experiments have been used to identify p53-responsive domains in the promoters of many cellular proteins. Of particular relevance to the

antiproliferative effects of wtp53 has been the demonstration that retinoblastoma gene expression is down-regulated by wtp53, and that the promoter of the gene encoding the so-called proliferating-cell nuclear antigen (an auxiliary factor of DNA polymerase δ) is also inhibited (Shiio *et al*, 1992; Deb *et al*, 1992). Both of these proteins probably play an important role in DNA replication, and their regulation by wtp53 may be partly responsible for the cell cycle block induced by the protein. However, in both these examples, direct binding of wtp53 to the promoter was not demonstrated: it is therefore possible that regulation of these and other promoters by wtp53 is, in fact, mediated by the binding of other cellular proteins whose activity has been affected, directly or indirectly, by the general change in the cycling state of the cell (Ginsberg *et al*, 1991).

Furthermore, it could be argued that p53 binding sites in genomic DNA are being used to control a process distinct from transcription, such as replication. Taken out of context in an artificial reporter gene expression vector, transcription may be affected as a result of a conformational change in the DNA, such as local unwinding of the helix, that is involved in a different process *in vivo*. Indeed, there is some evidence that implicates wtp53 directly in the process of DNA replication. First, immunostaining with anti-p53 antibodies has localised the protein to sites of viral replication in herpes virus-infected cells (Wilcock and Lane, 1991). Second, it has been demonstrated that wt, but not mutant, p53 can inhibit DNA replication from the SV40 origin of replication (Braithwaite *et al*, 1987; Sturzbecher *et al*, 1988; Wang *et al*, 1989; Friedman *et al*, 1990). This appears to be achieved by the combination of a number of different mechanisms, all involving inhibition of the normal function of the large T antigen. During initiation of viral replication, the large T antigen binds to the origin and unwinds the DNA. wtp53 binds to a sequence adjacent to the origin as a large oligomeric complex, where it prevents the binding of large T (Bargonetti *et al*, 1991). This region is similar to the p53-binding consensus, shown above, that acts as an transcription enhancer element. Large T is able to displace the wtp53 molecules from the DNA, but, once complexed, the wtp53 inhibits the DNA helicase activity of

large T and prevent it from binding to DNA polymerase α, properties required for large T to initiate normal viral replication (Sturzbecher *et al*, 1988; Gannon and Lane, 1987). To overcome these problems large T inactivates wtp53, thus removing inhibition at the viral, and also possibly at cellular, origins of replication.

More recently however, Zambetti *et al* (1992), identified a p53 binding site in the promoter of muscle creatine kinase which mediates up-regulation of the gene. This directly correlated p53-DNA interaction with transactivation, and since this discovery numerous other genes have been identified that are repressed or activated by wtp53 (Kastan et al, 1992; Chin *et al*, 1992; Agoff *et al*, 1993): one of these, *GADD45*, is discussed below (section 4.9). Moreover, a number of reports have proposed that wt, but not mutant, p53 may exert a general repressive effect on genes regulated by TATA boxes, by interacting directly with the basal transcription machinery (Seto *et al*, 1992; Mack *et al*, 1993; Ragimov *et al*, 1993). Thus, wtp53 has been accepted as a transcription factor, but this evidence does not exclude the possibility of a separate direct interactive role for wtp53 in the replication machinery.

4.7: Control of wtp53 function.

Interestingly, *in vitro*-translated p53 protein is unable to bind to its recognition sequence unless it is mixed with nuclear extract, a result which implied that post-translational modification, or possibly hetero-oligomer formation is required to activate DNA binding (Funk *et al*, 1992). Many potential phosphorylation sites have been identified in the amino- and carboxy-termini of the protein where the transactivation and oligomerisation domains are found (Figure 4.1), and thus, phosphorylation may modulate their activity. Serine 389 of mouse p53 (serine 391 in human p53), the penultimate amino acid of the protein, is phosphorylated by casein kinase II, a heterodimeric protein that is thought to mediate signal transduction of extracellular mitogenic stimuli (Meek *et al*, 1992). Mutation of this residue to an alanine removes the antiproliferative properties of wtp53 (Milne *et al*, 1992a), and phosphorylation of this serine appears to be required to permit wtp53 to bind to its

recognition site (Hupp *et al*, 1992). Similarly, deletion of the last thirty amino acids from the carboxy-terminus of the unphosphorylated protein allows DNA binding *in vitro*, identifying this region as a negative regulator of wtp53 function that can be controlled by phosphorylation (Hupp *et al*, 1992): larger deletions of this region abrogate DNA binding (Foord *et al*, 1991). The oligomerisation domain, thought to be essential for wtp53 function, is located in the negative regulatory domain identified by Hupp *et al* (1992).

These results have collectively given rise to the following hypothesis concerning the role of the carboxy-terminus in regulating p53 function. This proposes that the O2 domain (residues 341-355; Figure 4.1) mediates wtp53 dimerisation, and is therefore probably essential for DNA binding, whilst the O1 region (residues 365-386) is involved in sequestering wtp53 into large inactive oligomers which can be disrupted by O1 removal or phosphorylation by casein kinase II at the penultimate serine. However, removal of O2 prevents DNA binding irreversibly, explaining the selection for truncation mutants in developing tumour cells (Hupp *et al*, 1992). Intriguingly, serine 389 is also the target for addition of a small 5.8S rRNA moiety, the function of which remains a mystery (Fontoura *et al*, 1991; Samad and Carroll, 1991).

Serine 309 in mouse p53 (315 in human) is a target for the p34cdc2 kinase, a protein that controls cell cycle progression. Interestingly, this residue lies in one of the nuclear localisation domains, and thus, may control subcellular localisation of p53 in a cell cycle-dependent fashion (Milner *et al*, 1990; Bischoff *et al*, 1990a; Addison *et al*, 1990). At the amino-terminal are several serines, adjacent to and within the minimal transactivation domain, that are phosphorylated by a casein kinase I-like protein, and can be dephosphorylated *in vitro* by protein phosphatase 2A (Wang and Eckhart, 1992; Milne *et al*, 1992b). The exact role of these modifications is unknown, but interestingly, a double-strand DNA-dependent kinase also phosphorylates these sites and, as discussed below, may modify p53 activity in response to DNA damage.

4.8: Mutant p53 proteins fail to bind to DNA.

Defining wtp53 as a regulable transcription factor made it important to determine the effect mutation of the gene has on its properties. First, in carboxy-terminal truncation mutants, the lack of the oligomerisation domain means these proteins are unable to form a tetrameric complex with DNA. Second, in many of the reports showing transactivation by wtp53, it was also demonstrated that a variety of mutant p53 proteins carrying a single amino acid change had lost this ability (Raycroft *et al*, 1990; Deb *et al*, 1992; Kern *et al*, 1992; Zambetti *et al*, 1992; Unger *et al*, 1992; Farmer *et al*, 1992). Furthermore, Yew and Berk (1992) showed that transactivation by the wtp53-GAL4 fusion protein was dominantly inhibited *in vivo* by p53 mutants or by the E1b-55kDa protein, presumably by the formation of inactive oligomers. Invariably the mutant proteins have lost their ability to bind to DNA (Kern *et al*, 1991a, 1991b; El-Deiry *et al*, 1992), but Raycroft and her colleagues (1990, 1991) showed that even if a full-length tumour-derived point-mutated *p53* cDNA is linked to the DNA binding domain of GAL4, it is still unable to activate transcription from a GAL4 operon. When the ts *p53* mutant is used in these fusion experiments, transcription only occurs at 37.5°C, when the mutant has the transformation-suppressing ability of the wt (Raycroft *et al*, 1991). Thus, point-mutated tumour-derived p53 genes code for proteins that are unable to bind to DNA and/or activate transcription. Interestingly, a mutant protein from an LFS family exhibited transactivation to the same extent as wt in the GAL4 assay system, although the DNA binding ability of this protein was not assessed (Raycroft *et al*, 1991).

The viral oncoproteins produce the same effect on p53 DNA binding properties as seen with point mutation: E6 by degrading the protein and large T and E1b-55kDa by disrupting the interaction (Farmer *et al*, 1992; Lechner *et al*, 1992; Yew and Berk, 1992; Mietz *et al*, 1992; Segawa *et al*, 1993). It was proposed that there may be a functional cellular homologue of the viral oncoproteins that could be regulated to prevent DNA binding in response to specific signals (Levine *et al*, 1991). One such candidate has recently been identified, termed mdm2, that forms a complex

with p53, inhibits its DNA binding and masks the amino-terminal transactivation domain of the protein (Momand *et al*, 1992; Oliner *et al*, 1993). This gene was originally identified by virtue of its amplification in a transformed mouse fibroblast cell line (Cahilly-Snyder *et al*, 1987), and has been shown to increase the tumorigenicity of cells in which it is over-expressed artificially (Fakharzadeh *et al*, 1991), presumably because it interferes with wtp53 function. Indeed, Finlay (1993) has shown that *mdm2* can prevent wtp53-mediated suppression of rat embryo fibroblast transformation. Furthermore, Oliner *et al* (1992) detected amplification of the gene in a third of human sarcomas tested. These tumour types often do not have *p53* mutations: it is probably not required if *mdm2* is over-expressed.

Thus, whether by mutation of the gene itself, infection with viruses containing wtp53-inactivating proteins or by *mdm2* amplification, wtp53 appears to be removed from a large variety of developing tumour cells. Until recently, this was thought to result in uncontrolled proliferation, by mimicking the effect of mitogenic stimuli. However, there are now indications that wtp53 only prevents cell division at certain times, in particular when cells have sustained damage to their DNA.

4.9: p53 and DNA damage.

Although wtp53 can block cell cycling when expressed at high levels, it does not seem to play a role in normal cell cycle control. Thus, in mice lacking both *p53* genes, and in humans with germ-line mutations of *p53* (LFS), development is normal although there is an increased susceptibility to developing tumours (Donehower *et al*, 1992; Malkin *et al*, 1990; Srivistava *et al*, 1990). It seems unlikely that this normal development is due to functional redundancy. This would imply that cells containing a *p53* mutation would have to acquire a second mutation in the gene responsible for the redundancy to allow the *p53* mutation to be favourable for tumour progression. In fact, evidence is now emerging that the wtp53 protein prevents the division of cells that have sustained DNA damage.

In 1984, Maltzman and Czyzyk found that treatment of fibroblasts with UV or UV-mimetic drugs induced an increase in the levels of p53 protein as a consequence of a post-translational event (Maltzman and Czyzyk, 1984), a result repeatable in normal human skin (Hall *et al*, 1993). More recently, Kastan and his colleagues (1991) showed the same effect with γ-radiation and correlated it to a G1-S block in the cell cycle. They were unable to produce the same results in cells harbouring mutant p53 proteins unless wt*p53* expression was artificially restored (Kuerbitz *et al*, 1992).

These alterations in the wtp53 levels at times of stress probably alters the transactivating properties of the protein: indeed, it has been demonstrated that expression of the *GADD45* gene at this time is dependent on wtp53 binding to an element within one of its introns, which presumably does not occur without the radiation treatment (Kastan *et al*, 1992). *GADD45* was originally identified as being specifically up-regulated in response to DNA damage induced by ionising radiation (Fornace *et al*, 1988; Papathanasiou *et al*, 1991). Its function is unclear, but it is thought to be involved in DNA repair and shows slight homology to a yeast DNA repair gene, *rad1* (Papathanasiou *et al*, 1991). In cells that have lost wt*p53*, *GADD45* is not induced and gene amplification potential is increased; this can be reversed by restoring the wt*p53* by introducing expression constructs containing the gene (Kastan *et al*, 1992; Yin *et al*, 1992; Livingstone *et al*, 1992). Interestingly, Kastan *et al* (1992) have also shown that p53 stabilisation and *GADD45* expression are not induced following irradiation of cells from ataxia-telangiectasia individuals. This inherited genetic disorder is characterised by an increased instability of the genome, with homozygotes often developing cancer early in life (Swift *et al*, 1991). This is not surprising if the p53 G1-S checkpoint is abrogated. This result has also led to the suggestion that the product of the ataxia-telangectasia gene(s) is an upstream regulator of wtp53 function. Further support for a role for wtp53 in reducing DNA damage comes from observation that the large T antigen of SV40 or HPV E6 converts cells to a state that permits gene amplification (Perry *et al*, 1992; Kessis *et al*, 1993).

Furthermore, fibroblasts from LFS individuals exhibit an increased genomic instability and are more likely to become tumorigenic than their normal counterparts (Bischoff *et al*, 1990b, 1991).

From these results the following working model was proposed and is depicted diagrammatically in Figure 4.2 (Lane, 1992). DNA damage induces an increase in wtp53 protein levels, and also possible alterations in its transactivating properties, which prevents the transition into the S phase of the cell cycle. This gives time for the damage to be repaired; wtp53 may also induce the expression of the appropriate genes to do this. Should the DNA fail to be repaired, then apoptosis may be induced to prevent the division of a damaged cell (Figure 4.2A) (Yonish-Rouach *et al*, 1991; Shaw *et al*, 1992; Ryan *et al*, 1993; Johnson *et al*, 1993). Mutating *p53* during tumour progression removes this G1-S checkpoint, thus permitting the accumulation of other mutations that contrive to prevent maturation and enhance proliferation (Figure 4.2B).

Exactly how the DNA damage is detected, how it is converted into a wtp53 stabilisation message and which kind of damage (e.g. DNA breaks or point mutation etc.) induces the effect is currently unknown, but is presumably the subject of intense investigation. However, the model has received support in studies of T cell apoptosis in normal mice compared to p53-null animals. In the absence of p53, the cells were unable to die in response to radiation treatment, unlike their normal counterparts, but were still responsive to agents which mimicked T cell receptor-mediated death (Lowe *et al*, 1993; Clarke *et al*, 1993). With reference to the mechanism of p53-mediated damage-induced apoptosis, it is interesting to note that two groups have identified a protein kinase, termed DNA-PK, that is active only in the presence of double-stranded DNA and which phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of the p53 protein (Lees-Miller *et al*, 1990, 1992; Carter *et al*, 1990). It seems possible that such a kinase may be regulated by damaged DNA, although a great deal more work is needed to evaluate this signalling pathway and its affect on wtp53. Another possibility is that the *mdm2* gene product acts as a p53

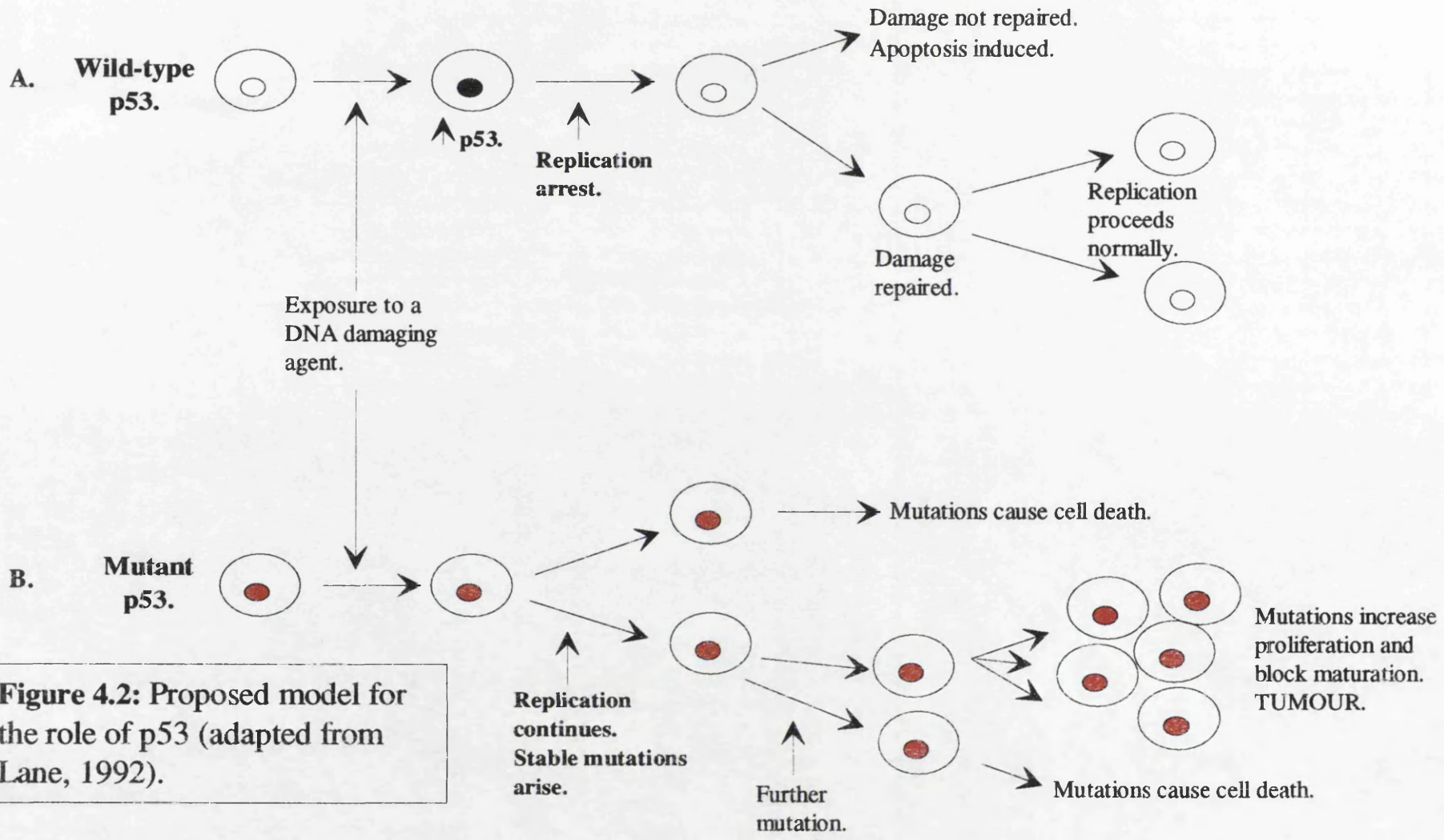


Figure 4.2: Proposed model for the role of p53 (adapted from Lane, 1992).

regulator that may be targeted by modifying enzymes, like protein kinases, to alter its affinity for p53.

4.10: Summary.

During the last three years, we have witnessed major advances in the understanding of the p53 tumour suppressor with the identification of its role as a transcription factor, the alterations in this property as a result of mutation, and the generation of a new working model for p53 as a DNA damage-regulated proliferation inhibitor. At the outset of this thesis, the effect of *p53* mutation on the progression of leukaemia was uncertain. Recent reconstitution experiments have attempted to link *p53* mutation with a loss of differentiation and/or a block in apoptosis. However, like other tumour cells, the genome of these cells is probably highly unstable, and these effects may be a consequence of damage detection, rather than a true representation of the role of wtp53 in normal erythroid progenitors. One problem with the Friend erythroleukaemia model is that only the most advanced leukaemic cells are available for study, which have lost most of the characteristics of normal erythroid progenitors. A cell system which shows a stepwise acquisition of particular phenotypes would facilitate attempts to correlate *p53* mutation with abrogation of a particular aspect of erythropoiesis. If, however, it is insufficient to affect these processes, cells should still be capable of differentiation and death in the absence of wtp53, a hypothesis supported by the normal development of p53-null mice.

CHAPTER 5: The ELM erythroleukaemia system.

5.1: Erythroleukaemia induced with X-ray irradiation.

A disease with the characteristics of an erythroblastic leukaemia, termed the ELM erythroleukaemia, was induced in a female C3H mouse by X-ray irradiation of 300 Rads when the animal was 10 weeks old (Figure 5.1) (Itoh *et al*, 1988a).

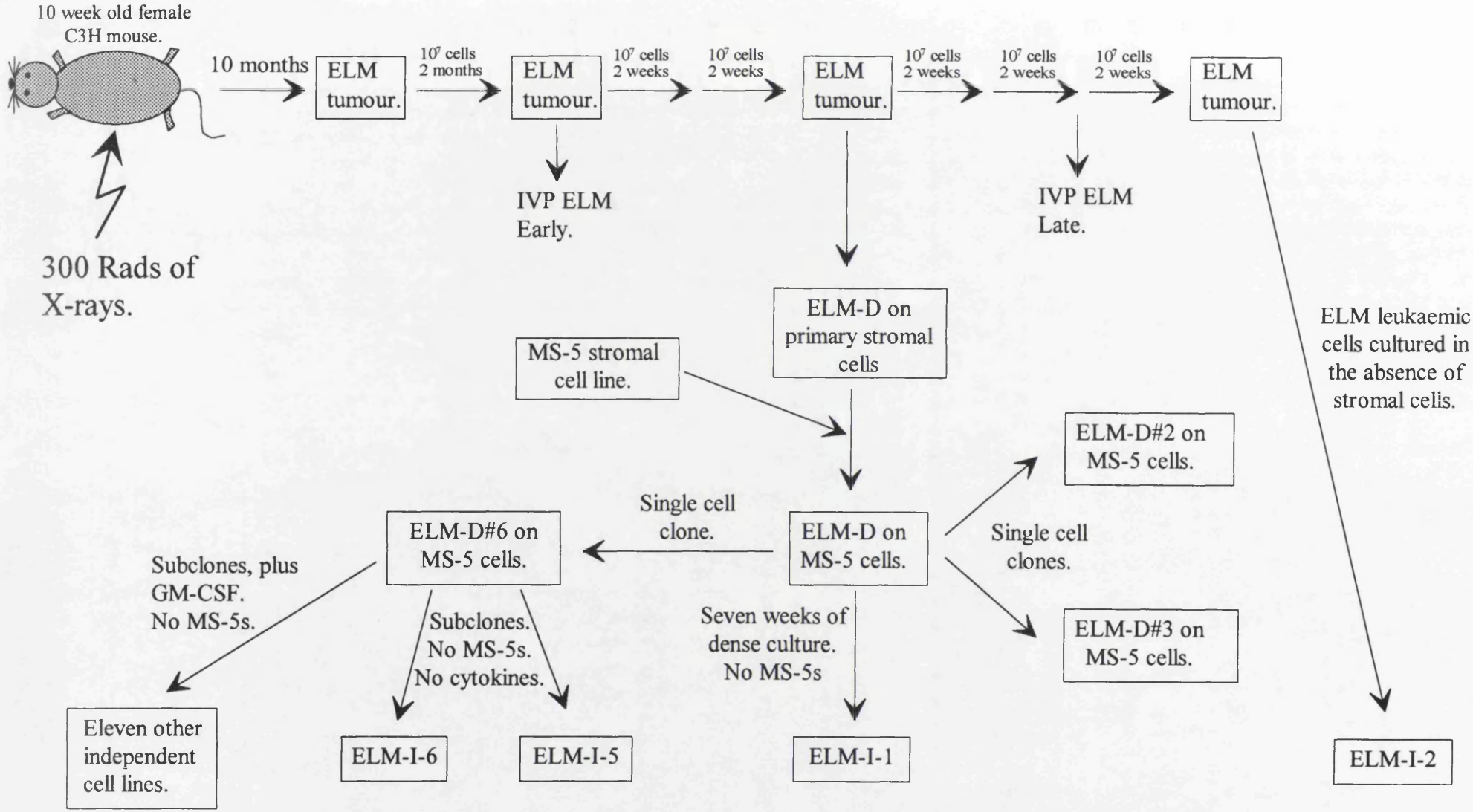


Figure 5.1: The ELM erythroleukaemia system.
(see text for further details).

Hepatomegaly, splenomegaly and anaemia were noticed after a ten month latency. Like Friend erythroleukaemia cells, *intra venous* injection of leukaemic ELM spleen cells induced the same disease in a syngeneic host although with a latency of two months. Subsequent passage *in vivo* generated erythroleukaemia within two weeks of injection, implying selection for more leukaemogenic cell clones during these early passages. Cytological analysis of peripheral blood from animals with a well-developed leukaemia showed that it consisted almost entirely of erythroid cells at different stages of differentiation.

It is unusual for irradiation to induce erythroid malignancies in mice; usually cells from the lymphoid and other myeloid lineages are affected, with the genetic background of the irradiated host determining, to some extent, the nature of the haematological malignancies formed (Janowski and Boniver, 1986; Mole, 1986). In some cases, these involve the production of recombinant endogenous retroviruses that are leukaemogenic in other non-irradiated host animals. Itoh *et al* (1988a) performed a number of experiments that appeared to exclude any endogenous or exogenous retroviral involvement in the ELM tumour. First, cell-free medium from short-term ELM spleen cell cultures, and ultrasonicated ELM cells, both failed to induce erythroleukaemia in 8-10 week-old C3H mice, demonstrating that no leukaemogenic retroviruses are being produced by the cells. Second, reverse transcriptase (RT) activity, which is present in retrovirus-producing cells, was not detected in the spleen and liver of mice with advanced ELM erythroleukaemia: the same assay had been used effectively to detect RT activity in Friend virus-infected cells (Ono *et al*, 1984). These results distinguished ELM from Friend and Rauscher virus-induced erythroleukaemias, and implied that the X-ray irradiation was the sole factor involved in initiating this malignancy, presumably by generating mutations in cellular genes. Autocrine growth factor production has been recorded in a number of haemopoietic malignancies (Miyanomae *et al*, 1985; Sawada *et al*, 1986; McDonald *et al*, 1987; Meeker *et al*, 1990). However, conditioned medium from short-term ELM leukaemic spleen cell cultures failed to increase erythroid 'burst' formation from normal bone

marrow cells, demonstrating that erythroid stimulatory factors, such as epo, were probably not being produced by the leukaemic cells as a result of these putative mutations (Itoh *et al*, 1988a).

5.2: Long-term growth *in vitro* supported by stromal cells.

Attempts to culture spleen cells from the ELM tumour *in vitro* proved difficult and even with IL-3, GM-CSF or epo supplements no cell lines could be established. This is quite distinct from Friend erythroleukaemias from which cell lines are readily generated, even in the absence of growth factors (section 2.3.4). However, after an initial decrease in viability, ELM cells grew well on stromal cells, initially on primary bone marrow-derived stromal cells, then later, on a bone marrow-derived stromal cell line, MS-5 (Figure 5.1) (Itoh *et al*, 1988b). This gave rise to the ELM-D cell line, the 'D' representing the stromal cell-dependence of the erythroleukaemia cells. The MS-5 cells are fibroblastic-like cells established after 900 Rads X-ray irradiation of a LTBMC; they are capable of supporting the adhesion-dependent growth of haemopoietic stem cells and their progeny for greater than two months in LTBMC (Itoh *et al*, 1989). The ELM cells adhere tightly to the stromal cells, with regions of extremely close contact (~10 Angstroms), and are physically separable only by trypsinisation (Itoh *et al*, 1993). Dependence on specific growth factors for the survival and proliferation of leukaemic cells *in vitro* has been demonstrated in malignancies affecting several other blood cell lineages (Poiesz *et al*, 1980; Lange *et al*, 1987; Spivak *et al*, 1990), but the use of stromal cells to support growth has rarely been recorded. However, Hiai *et al* (1981) showed that the proliferation of murine lymphoid leukaemia cells *in vitro* could be supported by stromal cells, an effect that was dependent upon intimate association between the two cell types. Likewise, Manabe *et al* (1992) and Gluck and his colleagues (Gluck *et al*, 1989) reported that human acute lymphoblastic leukaemia cells survived in the presence of stromal cells, but underwent apoptotic cell death when separated. Furthermore, recent unpublished work performed in Dr. Ostertag's laboratory suggests that coculture of murine

retrovirus-induced myeloid leukaemia cells with stromal cells dramatically increases the survival rate of the leukaemic cells *in vitro* (Ostertag, personal communication). Paradoxically, in 50% of acute myeloid or lymphoblastic leukaemias in humans, maintenance in LTBMCs has been used to select for normal cells and purge their leukaemic counterparts (reviewed in Dexter *et al*, 1990). This suggests that rather than supporting growth, stromal cells actually compromise leukaemic cell growth. The reasons for this phenomenon are unknown, but it demonstrates that during the progression of some leukaemias, but not others, cells develop altered survival requirements which are unavailable from normal LTBMC stromal cells.

The ELM-D/MS-5 cell line provides a unique opportunity to examine the interaction between stromal cells and erythroid progenitors. However, use of this system may not accurately reflect the situation *in vivo*. For instance, the ELM-D cells are capable of proliferation on the MS-5 stromal cells: one function of haemopoietic stroma may be to keep progenitor cells dormant and provide long-term survival, but not proliferation, until demand for a specific cell type arises. Furthermore, the ability of the ELM cells to generate leukaemias *in vivo* obviously indicates a loss of certain normal progenitor characteristics. However, the coculture may give an insight into cell-cell interactions involved in haemopoiesis. Thus, a collaborative study was initiated with Dr. Ostertag's group in Hamburg to study the coculture to attempt to define the cell biological characteristics of the interaction, and to identify stromal cell-derived signals that act to permit ELM-D cell survival and proliferation. The results of this study (Itoh *et al*, 1993) are described in part in sections 5.3 and 5.4 of this introduction, and also in the Discussion section, whilst my contribution is shown in the Results (Chapter 9).

5.3: Dependence on stromal cell contact for the long-term growth of ELM-D#6 cells.

The ELM-D cell line is a fairly heterogeneous population of ELM cells, containing a small proportion that will grow without stromal cells (Ostertag, personal

communication). In order to study the interaction between the stromal cells and the erythroleukaemia cells, several stromal cell-dependent ELM clones were generated from the original ELM-D cell line onto MS-5 cells, and termed ELM-D clone 2, 3 and 6, and referred to hereafter as D#2, D#3 and D#6 (Figure 5.1). The majority of the work described in this thesis used the D#6 cell line, although the other clones were used to confirm certain conclusions.

Clonal growth of D#6 cells in the presence of stromal cells was very efficient, but could be reduced by a factor of 10^3 when a stromal cell layer was omitted from the cloning assay dish. Furthermore, analysis of 200 D#6 colonies formed in this second experiment showed that they all contained contaminating stromal cells carried over from the coculture from which the D#6 cells were harvested. To get a true estimate of the cloning efficiency of D#6 cells in the absence of stromal cells, they were established on a lethally-irradiated stromal cell layer that supports D#6 growth for up to two weeks, but then disintegrates. When the cloning experiment was repeated, the survival rate of the D#6 cells was extremely low, with stromal cell-independent clones arising at a frequency of $<2x10^{-5}$ (section 5.4). However, during the first couple of weeks many large colonies were generated that later disintegrated. This was not due to stromal cell contamination which was undetectable, or due to the cells reaching saturation density since serial recloning of small colonies every two weeks resulted in clonal extinction after the second or third recloning stage. From these experiments, it was concluded that D#6 cells showed a exquisite dependence on stromal cells for long-term growth and survival, but were capable of proliferative cell expansion in the short-term (2-3 weeks). Furthermore, using an elegant two-layered agar culture system, it was shown that only when direct contact was established between D#6 cells and stromal cells adhered to a solid surface, was the full long-term viability of the leukaemic cells achieved. Moreover, MS-5 and MS-5/D#6 conditioned media were unable to replace the MS-5 cells in these assays.

The process by which the D#6 cells lose their viability once they are removed from the stroma remains uncertain. Differentiation is not observed and late erythroid

markers are not expressed; nor are the cells undergoing senescence, the process of irreversible proliferation arrest (reviewed in Goldstein, 1990), since cell number decreases dramatically once proliferation has ceased. However, it has proven difficult to demonstrate certain characteristics of apoptosis or programmed cell death, such as DNA fragmentation (Ostertag, personal communication), but this does not necessarily exclude this process for a number of reasons. First, it seems unlikely that all the D#6 cells die at the same time, which may make it difficult to detect apoptosis due to contamination by a large number of cells that have not yet initiated the process. Second, a recent report suggests that programmed cell death can occur using mechanisms quite distinct from the classically defined apoptotic pathway involving membrane blebbing, chromatin condensation and DNA fragmentation (Schwartz *et al*, 1993). A tentative conclusion is that D#6 cells undergo some form of programmed cell death once separated from the MS-5 cells, although possibly not via a classical apoptotic pathway (Itoh *et al*, 1993).

Several growth factors (IL-3, GM-CSF and soluble SCF; reviewed in Chapter 1) were tested to see if they could replace the stromal cells and support growth of D#6 cells. Whether alone, or in combination, long-term growth was never achieved, although the cells exhibited an increased life-span and a more extensive proliferative expansion than that seen in the complete absence of these growth factors and stromal cells. Thus, these ligands delayed, but did not prevent, the death of D#6 cells. Intriguingly, cells maintained initially in growth factors, then transferred back to stromal cells rapidly regained the full growth potential of cells maintained permanently on stromal cells. For example, GM-CSF permits D#6 proliferation for approximately eight weeks. If, after six weeks in GM-CSF, the cells are replated on MS-5 cells for as little as 48 hours (Ostertag, personal communication), they are then able to grow in GM-CSF without stromal cells for a further eight weeks before dying. This effect can be repeated time and time again, provided the cells are replated on stromal cells before the end of the crucial eight week period. It appears, therefore, that the MS-5 stromal cells are able to 'prime' the D#6 cells for growth factor-

dependent growth. Interestingly, GM-CSF and IL-3 are able to over-ride the survival signal from the stromal cells: treating a D#6/MS-5 coculture with these growth factors induces clonal extinction within the same time frame as D#6 cells in growth factor but in the absence of stromal cells (Itoh *et al*, 1993). *In vivo*, normal erythroid progenitor cells, lacking the increased proliferative capacity of the D#6 cells, may be subjected to a similar effect by erythropoietic growth factors to initiate maturation away from the survival signals emanating from the stroma, which manifests as apoptosis if the appropriate differentiation signals are absent.

The D#6/MS-5 coculture is perhaps analogous to the way in which antigen-dependent T-cells primed by antigen-presenting cells, can be maintained only temporarily in the presence of interleukin-2 after which they undergo apoptosis, and require renewed contact with the antigen-presenting cell for long-term growth and viability (Smith, 1988; Ramsdell and Fowlkes, 1990; Lenardo, 1991). Moreover, the observations discussed here using the D#6/MS-5 may be a general phenomenon in haemopoietic/stromal cell interactions. Human erythroid TF-1 cells, growing on stromal cells, exhibit similar properties as D#6 cells: retrovirus-induced leukaemic precursor cells from other myeloid lineages can also be 'primed' for growth factor-dependent proliferation by MS-5 stromal cells (Ostertag, personal communication).

5.4: Loss of stromal cell-dependence.

During the course of investigations by Dr. Ostertag's group, it was calculated that stromal cell-independent cells arise from the coculture at a frequency of $<2x10^{-5}$, a rate consistent with a requirement for a stable genetic, rather than epigenetic, alteration. Fifteen cell lines have been established from ELM-D and D#6 as a result of this phenomenon (Figure 5.1). ELM-I-1 was derived from the non-adherent cell population from a dense culture of ELM-D cells grown on feeders for seven weeks; the 'I' denotes that growth of these cells is independent of stromal cells and growth factors (Itoh *et al*, 1988b). ELM-I-2 arose after culturing ELM tumour cells (that had been passaged *in vivo*), directly *in vitro* in the absence of stromal cells. The

remaining ELM-I cell lines were independently derived from D#6 by selection for growth in suspension in the presence (eleven cell lines), or absence (I-5 and I-6), of GM-CSF. However, continued growth of all these cell lines proved not to be dependent on GM-CSF once feeder-dependence was lost (Ostertag *et al*, 1992). In this thesis, the cell lines I-1, I-2, I-5 and I-6 were routinely used, but the other cell lines were occasionally used to check the validity of certain conclusions.

5.5: Aims and objectives.

The ELM system is distinct from previously described murine erythroleukaemia systems in that a stromal cell-dependent cell line is available from which Friend cell-like cells arise with further mutation. This provides an excellent system in which to attempt to define (a) stromal factors required for the growth of erythroid progenitor cells and the nature of stroma/progenitor interaction, and (b) the genetic alterations necessary to generate distinct leukaemic cell phenotypes.

The first aim of this thesis was to further characterise the ELM system by examining the tumorigenicity and differentiation properties of the various cell lines. It seemed possible that this may demonstrate other phenotypic variations within the system. The second point of interest concerned the nature of the stromal cell-derived signal which allows the long-term growth of the ELM cells *in vitro*. This was undertaken as a collaborative project with Dr. Ostertag's laboratory, and aimed to first identify growth factors present in MS-5 cells, to verify the expression of the relevant receptors on the ELM cells, and then to examine the effect of abrogating the interaction between ligand and receptor on ELM cell survival. There were a large number of potential candidates, discussed in Chapter 1, but one particularly attractive possibility was SCF. This factor has a potent effect on erythroid progenitor cell growth, and, although soluble SCF does not permit D#6 cell survival, when it is presented as a transmembrane (TM) form it may act as a more permanent stimulus, a point raised and discussed in section 1.3.6. Alternatively, a TM SCF/c-kit interaction may mediate cell-cell adhesion (Flanagan *et al*, 1991) allowing the establishment of

close cell contact and other advantageous receptor:ligand interactions. In this scenario, SCF would be necessary, but not sufficient for ELM cell survival.

The third objective, and the main thrust of this thesis, was to identify genetic alterations involved in (a) generating the initial erythroleukaemia, and (b) changing the phenotype of the leukaemic cells throughout the system. The first genes to be examined were *p53* and the *ets* genes, *Spi-1* and *Fli-1*, known to be central to Friend virus-induced erythroleukaemia development (section 2.3.5). It was of interest to see if the same genes were involved when X-rays are used to induce leukaemia, rather than retroviral infection. With relation to the phenotypic differences between ELM cells, it is worth considering that the *wtp53* gene has been implicated in the induction of cell death in a number of cell lines, including Friend cells (section 4.2), and so its mutation may correlate with stromal cell-independent growth. Similarly, *ets* genes are thought to be nuclear components of mitogenic signal transduction pathways, and it is possible to envisage a situation where deregulated expression of an *ets* gene in a stromal cell-dependent cell could allow it to lose its requirement for the stromal cell-derived survival signal.

PART 2: METHODS AND MATERIALS.

CHAPTER 6: Methods.

6.1: Cell culture and differentiation induction.

6.1.1: Cell culture.

All the ELM cell lines (Itoh *et al*, 1988a), and the MS-5 stromal feeder cells (Itoh *et al*, 1989), were maintained in Minimal Essential Medium (α-MEM), containing deoxyribonucleosides and ribonucleosides, and supplemented with 20% (v/v) donor horse serum, 4mM glutamine, 100 μg/ml streptomycin and 37.5 μg/ml penicillin in 5% (v/v) CO_2 at 37°C. This medium will be referred to as ELM growth medium hereafter. F4-12 cells, which were derived from an SFFV-induced erythroleukaemia (Ostertag *et al*, 1972), were grown in Special Liquid Medium containing 10% (v/v) foetal calf serum, plus the same concentrations of glutamine and antibiotics used above, in 5% CO_2 (v/v) at 37°C. This cell line, and all the stromal cell-independent cell lines of the ELM system, grow to some extent adhered to the surface of the tissue culture flask, and were passaged as follows. Cells growing in suspension were harvested by centrifugation at 1,200 rpm for 5min in a MSE Centaur benchtop centrifuge. The remaining adherent cells were washed with PE, then removed from the flask by treating with a 0.25% trypsin solution in CT buffer, after which an equal volume of ELM growth medium was added to inhibit the trypsin. These cells were then harvested by a similar centrifugation step, and then combined with the suspension cells in 15ml of medium. 1-2ml was then used to seed a flask containing fresh medium.

ELM-D, D#2, D#3 and D#6 cells growing on live MS-5 cells, and the MS-5 cells themselves, were passaged in a similar way, except any suspension cells were aspirated from the flask and discarded. Reseeding the coculture at low cell number increased the ratio of leukaemic to stromal cells, and was employed to reduce stromal cell contamination in DNA, RNA and nuclear protein samples prepared from these cells (section 6.6). D#6 cells were also established on MS-5 cells irradiated with 17,000 Rads of γ-rays from an Alcyon II Teletherapy Unit containing a ^{60}Co source

(Ostertag *et al*, 1993). On passage, 10% of the cells from a freshly-irradiated confluent culture of MS-5 cells were added to 10% of the cells from a confluent ELM-D#6/MS-5 (irradiated) culture.

6.1.2: Cell storage.

Stocks of cells used in this work were stored in liquid nitrogen. Cells were harvested as above, resuspended to a final concentration of 10^7 cells/ml in ELM growth medium containing 10% (v/v) DMSO, then dispensed into 1-2ml Nunc cryotubes and stored at -70°C for 24hrs in a well insulated container. Tubes were then transferred to liquid nitrogen for long-term storage.

Cells were placed in a 37°C water bath to thaw, then diluted in 20ml of ELM growth medium and harvested by centrifugation for 5min at 1,200 rpm in a MSE Centaur benchtop centrifuge. Cells were then seeded into a tissue culture flask containing fresh medium.

6.1.3: Differentiation induction.

Cells were grown to 90% confluence, harvested, washed in phosphate-buffered saline (PBS) and then seeded at $2.5x10^5$ cells/ml in Ham's F12 medium (supplemented with the same components added to the ELM growth medium above), plus either no additional growth factors or 2U/ml of recombinant human epo and/or 10U/ml of murine IL-3. Cultures were incubated for 3, 5 or 7 days and the RNA extracted (section 6.6).

6.2: Animal experimentation.

6.2.1: Passage of ELM tumour cells and tumorigenicity assay.

Cells from a homogenised spleen, enlarged with ELM leukaemic cells, were provided by Dr. Ostertag. These cells, termed IVP ELM (late), had already been passaged several times through syngeneic C3H mice (see Figure 5.1 in the Introduction). These could be grown *in vivo* by resuspending $5x10^6$ cells in 0.2ml of

PBS and injecting them, via the tail vein, into recipient C3H mice immunosuppressed by irradiation with 300 Rads of γ-rays from a ^{60}Co source. After 14 days, the mice were sacrificed and the enlarged spleen removed and homogenised. Cells were then either passaged again *in vivo*, stored in liquid nitrogen in 10% (v/v) DMSO, or used to prepare RNA or DNA. Dr Ostertag also provided cells from an earlier passage of the ELM leukaemia, termed IVP ELM (early) (see Figure 5.1). RNA was extracted directly from these cells (section 6.6) to test for *Fli-1* and *erg* expression (see Results).

To test the tumorigenicity of the ELM cell lines, cells harvested from tissue culture were injected at the appropriate concentration into groups of female C3H mice, as above. After a specified latency period, by which time some animals appeared unhealthy, the mice were sacrificed, and the spleen and liver isolated and weighed, after first removing any visible connective tissue from the organ. A group of five control mice of similar age, which had not been injected with cells, were also sacrificed and their spleens and livers weighed.

6.2.2: Anaemia induction.

To generate erythropoietically-active spleens, anaemia was transiently induced in two C3H mice, by two consecutive injections of phenylhydrazine (60 mg/kg of body weight) separated by a 24hr period. 72hrs from the second injection, the animals were sacrificed, the spleens isolated and RNA extracted (section 6.6).

6.3: Recombinant DNA techniques.

6.3.1: Host cells.

Throughout this thesis, four separate strains of *E. coli* were used. In general, plasmids were propagated in JM83 cells (Genotype: *ara* Δ(*lac-pro*AB) *rps*L ϕ80*lacZ*ΔM15). Occasionally, when high transformation was required, library-efficient competent DH5α cells, purchased from GIBCO, were used (Genotype: *supE*44 Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1).

Propagation of *erg*-containing plasmids used the *INVαF'* cells (Genotype: *endA*1 *recA*1 *hsdR*17 (r^-_K,m^+_K) λ^- *supE*44 *gyrA*96 *thi*-1 *relA*1 ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR*) that were provided with the TA cloning kit (see Materials). Preparation of the genomic library used the P2 lysogen bacterial strain, P2392 (Genotype: *supE*44 *supF*58 *hsdR*514 *galK*2 *galT*22 *metB*1 *trpR*55 *lacY*1 P2), which will only permit the growth of recombinant bacteriophages lacking the *red* and *gam* genes from the stuffer fragment.

JM83, DH5α, *INVαF'* and their derivatives were grown at 37°C shaking at 250rpm in a New Brunswick G25 shaker at 37°C in Terrific broth or L-broth liquid medium, or on inverted 1.5% (w/v) agar L-broth plates also at 37°C, in both cases supplemented with the appropriate antibiotics. Ampicillin and kanamycin were used at a final concentration of 50μg/ml. P2392 cells were grown under similar conditions in NZCYM liquid medium or on 1.5% (w/v) agar NZM plates. A description of how these cells were used is included in section 6.16 concerning preparation and screening of the genomic library.

6.3.2: Preparation of competent cells.

DH5α and *INVαF'* cells were purchased in a transformation-competent state. To prepare competent JM83 cells, 2ml of an overnight culture was used to inoculate 200ml of L-broth (plus the necessary antibiotics), which was then incubated at 37°C shaking at 250rpm until the absorbance at 650nm was 0.5 units above an L-broth blank. These log. phase cells were harvested by centrifugation at 2,000rpm for 10min at 4°C in an IEC Centra-8R centrifuge. The pelleted cells were resuspended in 80ml of RF1 solution (100mM RbCl, 50mM $MnCl_2$, 30mM KAc, 10mM $CaCl_2$, 15% (v/v) glycerol, pH5.8), incubated on ice for 2hrs, then pelleted with an identical centrifugation step and resuspended in 16ml of RF2 solution (10mM MOPS, 10mM RbCl, 75mM $CaCl_2$, 15% (v/v) glycerol). After a 15min on ice, 0.8ml aliquots were transferred into 2ml Nunc cryotubes, frozen in a dry ice/ethanol bath, then transferred to -70°C for storage.

6.3.3: Transformation of bacterial hosts.

Competent cells were thawed slowly at 4°C, and 100µl aliquots put into precooled 15ml 2059 Falcon tubes. An appropriate amount of plasmid DNA or ligation mix (<10ng) was pipetted into the cells, mixed by gently tapping the tube, and incubated on ice for 30min. The cells were then heat-shocked at 37°C for 45sec, cooled on ice for 2min and then 0.9ml of L-broth added. This was incubated for 1hr at 37°C, shaking at 225rpm in a New Brunswick G25 shaker, to allow time for the expression of the antibiotic resistance gene. 100-200µl was spread onto 1.5% (w/v) agar L-broth plates supplemented with the appropriate antibiotic. The plates were allowed to dry for 5min, then inverted and incubated overnight to allow colony formation. To identify colonies containing recombinant plasmids in which the *lacZ* gene of the vector had been disrupted, blue/white colour selection on X-gal was often employed. Prior to spreading the cells, 40µl of a 20µg/ml solution of X-gal, mixed with 160µl of L-broth, was spread onto the plate and left for 1hr to permit full absorption into the agar. White colonies will contain a non-functional *lacZ* gene.

6.3.4: Bacterial glycerol stocks.

Host strains, and their derivatives containing useful plasmids, were stored as glycerol stocks for future retrieval. Stationary cultures in liquid medium were mixed with an equal volume of a 30% (v/v) glycerol/L-broth solution, cooled on ice, then frozen at -70°C. Cells were retrieved using a sterilised tungsten loop.

6.4: Isolation of plasmid DNA.

6.4.1: Purification and precipitation of nucleic acid samples.

Unless otherwise stated, these procedures were performed as follows. To remove protein contaminants, a combination of phenol-only (ϕOH), phenol/chloroform (ϕOH/$CHCl_3$) (50:50 (v/v) mix) and chloroform ($CHCl_3$) extractions were used. Phenol was prepared according to Sambrook *et al* (1989). $CHCl_3$ was saturated with distilled water. To extract proteins, an equal volume of

these solutions was added to nucleic acid samples, vortexed, and the layers separated by centrifugation either for 10min at 3,000rpm at RTemp in an IEC Centra-8R rotor (large samples), or for 5min at 13,000 at RTemp in a MSE Micro Centaur (small samples), hereafter referred to as a microfuge. The upper layer was invariably the nucleic acid-containing fraction.

Nucleic acid was precipitated by the addition of 2.5 volumes of ethanol and then NaAc (pH5.2) to a final concentration of 0.1M. This was mixed by vortexing, cooled to -70°C for 1hr, and the nucleic acid pelleted by centrifugation for 25min at 3,000rpm at 4°C in an IEC Centra-8R rotor (for large samples), or for 15min at 4°C in a microfuge.

6.4.2: Minipreparations of plasmid DNA.

Colonies were picked from an agar plate with a sterile toothpick and used to inoculate 10ml of Terrific broth (containing appropriate antibiotics), which was incubated overnight at 37°C shaking at 250rpm. Cells from 1.5ml of this culture were pelleted by spinning for 1min in a microfuge, resuspended in 100µl of solution A (50mM glucose, 25mM Tris.Cl (pH8), 10mM EDTA, 4mg/ml lysozyme) and stored on ice for 10min. 200µl of alkaline lysis buffer (1% SDS, 0.2M NaOH) was added, mixed gently, and incubated on ice for 15min to allow the bacteria to lyse. 150µl of 3M NaAc (pH5.2) was mixed into the preparation by vortexing, and placed on ice for a further 5min. Cell debris was removed by a 5min spin in a microfuge and the supernatant ϕOH/$CHCl_3$ extracted to remove residual protein. Plasmid DNA was precipitated by the addition of 1ml of ethanol and incubation at RTemp for 5min, then pelleted by a 10min centrifugation in a microfuge. DNA was resuspended in 40µl of 1xTE (10mM Tris.Cl (pH7.5), 1mM EDTA), and 10µl used in restriction digests.

6.4.3: Large scale preparations of plasmid DNA.

The alkaline lysis protocol, employing the same solutions, was also used to purify large amounts of plasmid DNA. All centrifugation steps in this protocol were

performed in 50ml 2098 Falcon tubes spun at 3,000rpm in an IEC Centra-8R rotor cooled to 4°C. 200ml of Terrific broth (containing the necessary antibiotics) was inoculated with 5ml of an overnight cell culture and incubated for 16hrs at 37°C. Cells were harvested with a 20min spin, 2ml of solution A added to each tube and the cells resuspended and left on ice for 10min. 4ml of alkaline lysis buffer was mixed gently into the cell suspension, left on ice for a further 20min to permit cell lysis, and 3ml of 3M NaAc (pH5.2) added. After another 5min on ice, cell debris was removed by spinning for 20min and passing the supernatant through a gauze swab. A two-thirds volume of isopropanol precipitated the DNA after 15min at RTemp, which was pelleted with a 40min spin. This was resuspended in 5ml of 1x TE, 0.5mg of DNAse-free RNAse was added and the mixture incubated at 37°C for 30min. After a ϕOH/$CHCl_3$ extraction, and an ethanol precipitation step, the plasmid DNA was loaded onto a Bio-Rad Bio Gel A-50M column in 1ml of 1x TE containing a one tenth volume of gel loading buffer (see section 6.5.1) and 2ml fractions eluted with 1x TE. 5µl aliquots from these fractions were run on a 1% (w/v) agarose gel (as described in section 6.5.2) to assess for the presence of DNA. Positive fractions were pooled, the plasmid precipitated with ethanol and resuspended in 0.5ml of 1x TE. The DNA was further purified by two ϕOH/$CHCl_3$ extractions, a $CHCl_3$ extraction and an ethanol precipitation, and finally resuspended in 0.2-0.5ml of TE.

6.4.4: Quantitation of DNA and RNA.

Nucleic acid concentrations were determined spectrophotometrically. 5µl of the sample was added to 495µl of distilled water and absorbance (A) readings taken at 260 and 280nm in a quartz cuvette compared to a distilled water blank. An A_{260} value of 1 was taken to be equivalent to 50µg/ml of plasmid or genomic DNA, 40µg/ml of RNA and 20µg/ml of oligonucleotide. The A_{260}/A_{280} ratio was used as a measure of purity: samples giving a ratio less than 1.75 were further purified by ϕOH/$CHCl_3$ extraction and ethanol precipitation, and the A_{260}/A_{280} ratio reassessed.

6.5: Restriction digests and agarose gel electrophoresis of plasmid DNA.

6.5.1: Restriction digests.

Restriction digests were carried out in small volumes buffered using concentrated solutions available with the enzyme from the supplier. Small quantities of plasmid DNA (<2μg) were digested in a total volume of 20μl using 1-10 units of enzyme per μg of DNA, depending on the enzyme used and the number of sites present. Larger, preparative digests were carried out in proportionately larger volumes. For double digests, suppliers' information was consulted and the appropriate buffer used. DNAse-free RNAse, at a final concentration of 0.5μg/μl, was added to digests of minipreparations of plasmid DNA. Reactions were incubated for 2-3hrs at the recommended temperature (usually 37°C), then terminated by the addition of one tenth volume gel loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 25% (v/v) Ficoll in water).

6.5.2: Agarose gel electrophoresis.

DNA fragments were resolved on non-denaturing agarose gels and visualised by ethidium bromide staining. In general, 1% (w/v) agarose gels were used, but smaller fragments (100-400bp) were separated on 2-4% gels. Preparative gels used low melting-point agarose at a concentration of 1% (w/v). Gel mixes containing the appropriate amount of agarose in 1xTAE buffer (50xTAE is 2M Tris, 50mM EDTA, 57.1ml/l glacial acetic acid) were heated in a microwave to dissolve the agarose, cooled to approximately 60°C, ethidium bromide added to 5μg/ml then poured into an appropriate gel former. Gels were allowed to set at room temperature, cooled for 10min at 4°C, and installed into the electrophoresis tank in 1xTAE buffer. Samples, containing one tenth volume of gel loading buffer (section 6.5.1), were loaded and separated at various currents. Molecular weight standards used include bacteriophage λ (*Hind* III-digested), bacteriophage ϕx174 (*Hae* III-digested) and the 1kb ladder. A photograph was taken of the gel with a Polaroid camera, using a UV source to illuminate the DNA bands.

6.5.3: Purification of DNA fragments from agarose gels.

Three different procedures were used depending on the size and quantity of the fragment of interest. In all cases, DNA was electrophoresed in low melting-point agarose and excised after visualising with ethidium bromide and a UV source. For fragments >500bp but <4kb in length, which were present in large quantities, 'Genecleaning' was used in accordance with the suppliers instructions. For fragments <500bp or >4kb, or when a higher recovery of the fragment was required, pre-packed NACS columns were employed. The columns were equilibrated and run at 37°C according to the manufacturers instructions, and the resulting purified fragment precipitated with ethanol in a solution containing 10μg of tRNA to act as a carrier. The pelleted DNA was resuspended in a small volume of 1x TE (≤30μl).

Electroelution of DNA from a gel slice was used only in the preparation of I-1 genomic DNA partially-digested with *Sau* 3AI during the construction of a genomic library (see section 6.16.3), due to the large size of the agarose fragment. The gel slice was enclosed in a piece of dialysis tubing containing 5ml of 0.5xTAE, and placed in a tank of 0.5xTAE. The DNA was electrophoresed from the agarose by applying 100V across the tank for 3hrs. The polarity was then reversed for 30sec, to remove the DNA from the tubing walls, after which the buffer was removed and spun at 3,000rpm in an IEC Centra-8R rotor for 5min to remove any agarose fragments. After a ϕOH/$CHCl_3$ and a $CHCl_3$ extraction, the DNA was precipitated from the supernatant using ethanol, and the pellet resuspended and further purified on a NACS column.

Prior to further manipulation, the quality and quantity of the purified fragment(s) was assessed by electrophoresing a small aliquot of the DNA on an agarose gel.

6.5.4: Ligation of DNA fragments into plasmids.

Both plasmid and potential insert were digested and purified as described above. The plasmid was dephosphorylated at its termini to prevent religation, by

including two units of calf intestinal alkaline phosphatase in the restriction enzyme digestion reaction. The DNA was then included in the following reaction mix in a final volume of 10µl:

~20ng of dephosphorylated plasmid

2-5-fold molar excess of the potential DNA insert

2µl of 5x ligation buffer (0.25M Tris.Cl (pH7.6), 50mM $MgCl_2$, 5mM dATP, 5mM dithiothreitol, 25% (w/v) polyethylene glycol-8000)

1µl of T4 DNA ligase (1 U/µl)

This was incubated at 16°C overnight and 1-3µl used to transform competent cells (see section 6.5.3).

6.6: Preparation of RNA and genomic DNA.

6.6.1: Preparation of cells and use of the elutriating centrifuge.

For the preparation of genomic DNA and cellular RNA, single cell suspensions of the sample were prepared. Cells in culture were harvested according to section 6.1.1. For the stromal cell-dependent cell lines, attempts were made to reduce MS-5 contamination by (i) only selecting cultures with a high leukaemic cell: stromal cell ratio, and (ii) harvesting cells by partial trypsinisation to release fewer MS-5 cells from the surface of the flask.

A more efficient protocol to separate stromal cell-dependent cells from MS-5 feeders was developed late in the preparation of this thesis, and was used to generate a nearly 100% pure population of leukaemic cells. This employed an elutriating centrifuge (Beckman Elutriator Rotor system), operated according to the manufacturers instructions and with the help of Dr. J. Lanfear. This separates cells with respect to their size by applying a flow-through of media against a centrifugal force. By adjusting the flow rate through the machine, cells of different sizes can be collected. The centrifuge was calibrated using pure populations of MS-5 or ELM-I-2 cells. D#6 cells were then collected from a coculture at a flow rate of 0.33ml/sec and 0.4ml/sec, rates at which I-2, but not MS-5, cells could be collected. $5x10^5$ cells were

seeded into 10ml of fresh medium and the proportion of leukaemic to stromal cells assessed visually: both preparations had a very low number of stromal cells. These cells were passaged *in vitro*, in the absence or presence of MS-5 cells, for several weeks to ensure their stromal cell-dependence. Other samples taken from the elutriating centrifuge at 0.33ml/sec were grown in ELM growth medium for 2-3hrs and RNA and nuclear proteins extracted. Tissue samples were homogenised in PBS in a Dounce homogeniser. All cell suspensions were harvested by centrifugation at 1,200rpm for 5min in a MSE Centaur benchtop rotor, washed with PBS, repelleted by a second spin and the supernatant removed: DNA and RNA was then extracted.

6.6.2: Preparation of mammalian genomic DNA.

Cells were resuspended in 1x TE to a final concentration of $5x10^7$ cells/ml, and 10ml of extraction buffer added per ml of cells (Extraction buffer: 10mM Tris.Cl (pH8), 0.1M EDTA, 0.5% (w/v) SDS, 20µg/ml DNAse-free RNAse). After a 60min incubation at 37°C mixing gently on a Stuart TR-2 tube rotator, proteinase K was added to a final concentration of 0.1mg/ml. This was gently mixed and placed in a 50°C water bath for 3hrs, swirling periodically. After cooling to RTemp, the protein fragments were removed by two phenol-only extractions. DNA was ethanol precipitated and collected by spooling it onto a clean Pasteur pipette moulded into a hook shape over a Bunsen. The DNA was allowed to air-dry for 15min then slowly resuspended at 4°C for three days, in a volume of 1x TE such that 1ml was included for every $2x10^7$ cells in the original sample. This solution was then dialysed in 1x TE for 18hrs at 4°C to remove any remaining salt and ethanol. The final solution was stored at 4°C: the DNA concentration was determined spectrophotometrically just prior to its use.

6.6.3: Preparation of mammalian RNA.

Total cellular RNA was prepared using the RNAzol B method, following the manufacturers instructions. However, once the RNA had been isolated, additional

$\phi OH/CHCl_3$ and $CHCl_3$ extraction steps were performed to further purify the sample, after which the RNA was precipitated with ethanol, washed in 70% (v/v) ethanol, resuspended in a small volume (<200µl) of RNAse-free water and stored at -20°C.

To avoid degradation by contaminating RNAses, a number a precautionary steps were taken. First, all tubes and solutions were precooled on ice, and all manipulations and centrifugations were carried out at 4°C. Second, all the tubes used were pretreated with diethylpyrocarbonate (DEPC), an irreversible inhibitor of RNAses. The tubes were immersed in a 0.1% (v/v) solution of DEPC overnight in a fume hood: the solution was then decanted off the tubes which were autoclaved, then dried in an 80°C oven. These were subsequently stored in a sealed DEPC-treated container. DEPC-treated water was also prepared by incubating a 0.1% (v/v) DEPC solution in a fume hood overnight then autoclaving. This was used in all the solutions employed in the protocol.

6.7: Preparation of oligonucleotides.

Oligonucleotides used in this thesis were synthesised on an Applied Biosystems model 381A DNA synthesiser according to the manufacturers instructions. 5' trityl groups were removed by the machine, and the DNA immobilised on a column. The DNA was eluted in 29% (v/v) ammonia by passing the solution through the column ~20 times every 10min for 1.5hrs. This solution was sealed in a glass vial and incubated at 55°C overnight. DNA was precipitated at -20°C for 2hrs in 2.5 volumes of ethanol in a final concentration of 0.1M NH_4Ac, then pelleted by centrifugation at 13,000rpm in a microfuge. The oligonucleotides were resuspended in 0.4ml of water, ethanol precipitated in the conventional manner (section 6.4.1), washed in 70% (v/v) ethanol, dried, and resuspended in 0.2-0.5ml of water. The concentration of the oligonucleotide solution was determined spectrophotometrically (section 6.4.4) and the sample stored at -20°C until required. The sequences of the oligonucleotides used in this thesis are shown in Tables 6.2, 6.3 and 6.4.

6.8: Northern blot analysis.

20μg of RNA was freeze-dried then resuspended and denatured at 65°C for 10min in 25μl of RNA loading buffer (1xMOPS/EDTA buffer [20mM MOPS, 5mM sodium acetate, 1mM EDTA (pH7)], 10% (w/v) formaldehyde, 50% (v/v) formamide, 0.5% (w/v) bromophenol blue). The samples were then cooled on ice, ethidium bromide added to a final concentration of 10μg/ml, then separated on a 1% (w/v) denaturing agarose gel containing 6.5% (w/v) formaldehyde in a 1xMOPS/EDTA buffer. A photograph was taken of the gel illuminated by a UV source. Gels were washed twice in sterile water for 20min then for 15min in 20xSSC (1xSSC is 150mM NaCl, 15mM sodium citrate) prior to vacuum blotting onto Hybond-N membrane, using 20xSSC as the transfer buffer. Filters were fixed by baking for 2hrs at 80°C. Prehybridisation was performed at 42°C for between 6-18hrs in 5xSSPE (1xSSPE is 180mM NaCl, 10mM NaH_2PO_4, 1mM EDTA), 5xDenhardt's (0.1% (w/v) bovine serum albumin, 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinyl pyrollidone), 0.5% (w/v) SDS, 50% (v/v) formamide and 50μg/ml heat-denatured herring testis DNA. Hybridisation was performed for 18-24hrs in the same solution containing probes radiolabelled with ^{32}P-labelled phosphate (described in section 6.10). Filters were washed twice at room temperature in 2x SSPE, 0.1% (w/v) SDS for 10min, then for 15min at 65°C in 1x SSPE, 0.1% (w/v) SDS and finally for 10min at 65°C in 0.1x SSPE, 0.1% (w/v) SDS. Filters were exposed to Kodak X-OMAT AR imaging film at -70°C. Some autoradiographs were scanned using a Molecular Dynamics ImageQuant version 2.0 computing densitometer. For each band the value of the OD multiplied by area of the band was calculated and used for comparative analysis of gene expression between RNA samples. Filters were stripped in boiling 0.1% (w/v) SDS prior to reprobing. Sizes of RNA species were determined by comparison with a RNA ladder run next to the samples, that was blotted and fixed as above, then cut off the blot and treated for 15min in 5% (v/v) acetic acid, 10min in RNA stain (0.5M NaAc, 0.04% (w/v) methylene blue), then washed in water to visualise the bands.

6.9: Southern blot analysis.

For most Southern blots, 20μg of DNA was digested for 16hrs, in the appropriate buffer and at the recommended temperature, with 50U of enzyme in a final volume of 90μl, then for another 6hrs after the addition of a further 20U of the enzyme. Digestion was stopped by the addition of 10μl of gel loading buffer (section 6.5.1). Digestions using large amounts of the *Dra* III restriction endonuclease were performed in a final volume of 250μl. After incubation at 37°C for 16hrs, *Dra* III-cleaved DNA fragments were ethanol precipitated and resuspended in 80μl of 1x TE plus 8ml of gel loading buffer (section 6.5.1).

Samples were then separated on a 0.9% (w/v) agarose 1x TAE gel run at 40mA overnight in 1x TAE buffer. The gel was then depurinated with a 15min treatment with 0.2M HCl, denatured in 1.5M NaCl, 0.5M NaOH for two spells of 15min, then neutralised for 1hr in 3M NaCl, 0.5M Tris.HCl (pH 7.5), changing the solution twice. Blotting, hybridisation, washing, autoradiography and densitometric scanning was carried out as for the Northern blots, except formamide was omitted from the prehybridisation and hybridisation stages which were, therefore, performed at 65°C. Probes were labelled with ^{32}P-labelled phosphate as described in section 6.10.

6.10: Random-primed radiolabelling of DNA probes.

All DNA probes used for hybridisation to Northern and Southern blots were labelled with [α-^{32}P] dCTP using a random-priming kit supplied by Boehringer Mannheim, in accordance with the manufacturers instructions. Unincorporated nucleotides were removed by gel filtration on a Sephadex G-50 column run in 0.1x SSPE, 0.1% (w/v) SDS. Radiolabelled probes were denatured by boiling for 5min prior to addition to the hybridisation mix.

Probes used in this study were either donated or generated during the course of the thesis. Table 6.1 gives the details, and appropriate reference, for all the probes generously given by other workers.

TABLE 6.1.

DNA Probes.

The following probes were obtained from other workers and are listed along with the appropriate reference. The figures adjacent to the restriction enzyme denote the position of the cleavage site, with numbering taken from the reference cited.

Gene.	Probe.	Source.	Reference.
Human *c-ets-2*	499bp *Sst* I$_{1152}$/*Ava* I $_{1651}$ cDNA fragment spanning most of the *ets* domain. Hybridises to murine *c-ets-2*.	A.Begue	Watson *et al*, 1988
Human *c-ets-1*	435bp *Pst* I$_{394}$/*Pst* I$_{829}$ cDNA fragment N-terminal to the *ets* domain. Hybridises to murine *c-ets-1*.	A.Begue	Watson *et al*, 1988
Murine *p53*	Full-length cDNA fragment (*Xho* I/*Bgl* II) excised from p4JJKan.	J.Jenkins	Jenkins *et al*, 1984
Murine SU-9	~800bp *Hinc* II/*ECo* RI genomic fragment from the *Fli-1* locus.	E.Rassart	Bergeron *et al*, 1991
Murine skeletal *Actin*	1177bp *Pst* I$_{2}$/*Pst* I$_{1175}$ cDNA fragment cleaved from the pAM91 plasmid.	F.Fee	Minty *et al*, 1982
Murine *GAPDH*.	720bp cDNA probe isolated by the RT-PCR protocol (section 6.11) by M. Walker.	M.Walker	Tso *et al*, 1985
F-MuLV *ENV*	830bp *Bam* HI$_{5718}$/*Bam* HI$_{6548}$ fragment spanning the *envelope* gene of F-MuLV.	W.Ostertag	Perryman *et al*, 1991
F-MCF *ENV*	486bp *ECo* RI$_{734}$/*Kpn* I$_{1120}$ fragment isolated from the pMCF-54B plasmid. Spans the *envelope* gene of F-MCF.	W.Ostertag	Koch *et al*, 1984 Oliff *et al*, 1983
SFFV *gp55*	620bp *Bam* HI$_{165}$/*ECo* RI$_{785}$ portion of SFFVp *envelope* gene, *gp55*.	W.Ostertag	Wolff *et al*, 1983
Murine alpha *Globin*	377bp Pst I358/Pst I735 genomic fragment spanning the first exon, and half of the second exon.	P.R.Harrison	Nishioka and Leder, 1979

6.11: Reverse transcription-polymerase chain reaction (RT-PCR).

The reverse transcription (RT) reaction was performed as previously described (Bartek *et al*, 1990), using 10μg of total cellular RNA. The gene-specific oligonucleotides used to prime the reaction are shown in Table 6.2A: *erg* RT reaction was primed with oligo-dT. 5μl from the reverse transcription reaction was amplified by PCR using the primers shown in Table 6.2B. The reactions contained 10μl of 10x reaction buffer (500mM KCl, 15mM $MgCl_2$, 100mM Tris.Cl (pH9), 1% (v/v) Triton X-100), 8μl of 2.5μM dNTPs, 500ng of each primer and 10U of Taq polymerase in a total volume of 100μl, overlaid with 100μl of paraffin oil. The reactions were incubated for 30 cycles at 93°C for 1min, 55-60°C for 1min and 72°C for 1-2min (depending on the length of the fragment being amplified). The products were electrophoresed on an agarose gel and the appropriate sized fragments purified on NACS columns, as previously described (section 6.5.3).

The *p53* PCR products were further purified by phenol/chloroform extraction and ethanol precipitation prior to direct sequencing (section 6.13). The *Fli-1* product was cloned, to create the p*Fli* vector, by cleavage of the *Hind* III and *Sal* I sites incorporated into the primers, ligation into *Hind* III/*Sal* I cut, alkaline phosphatase-treated pBluescriptII KS+, and selection on ampicillin/X-gal plates (section 6.5.4). The *Spi-1* cDNA fragment was cloned using *Xho* I and *Hind* III restriction sites in the primers. Likewise, the *SCF*, *c-kit* and *epoR* PCR fragments were inserted into the pBluescriptII KS+ vector by virtue of restriction sites *Xba* I/*Bam* HI, *Pst* I/*Hind* III and *Pst* I, respectively, included in the oligonucleotide primers. The *erg* PCR product was cloned using the TA cloning kit, according to the manufacturers instructions.

6.12: PCR from genomic DNA.

Amplification of *p53* genomic fragments used 0.5μg of DNA as a template and the primers shown in Table 6.3A. The oligonucleotides *p53* U2, *p53* I25' and *p53* I23' will not prime the *p53* pseudogene on account of their 3' end terminating in intron sequences, thus allowing preferential amplification of the correct genomic fragment.

TABLE 6.2.

A. Oligonucleotide primers for reverse transcription.

Name.	Sequence.	Position.
p53 RT	5' CAGCAGAAGGGACCGGGAGGATTG 3'	1247-1270
Fli-1 RT	5' TCCAGTTTTCTTATCTCTTC 3'	1687-1706
SCF RT	5' TTAGGATCCACATGAACTGTTACCAGCC 3'	1042-1062
c-kit RT	5' GAGAGAAAGCTTGGGGTCGGAGACAGCAGCAAA 3'	2980-3000
Spi-1 RT	5' GAGAGAGAATTCCTGGCGGTCTCTGCGGGCG 3'	953-972
EpoR RT	5' GGCCAGATCTTCTGCTGCAGAGTC 3'	853-875

B. Oligonucleotide primers for PCR from reverse-transcribed mRNA.

Name.	Sequence (plus restriction enzyme site).	Position.
p53 D1	5' CAGCAGAAGGGACCGGGAGGATTG 3'	1247-1270
p53 U1	5' ACCTCACTGCATGGACGATCTGTTGCT 3'	111-137
p53 D2	5' CTATTACACATGTACTTGTAGTGGATG 3'	684-710
p53 U2	5' TTCCACCTGGGCTTCCTGCAGTCT 3'	316-339
Fli-1 D1	5' GAGAGAGTCGACAGAGTATCCAGTAAAGTGAGA 3' (*Sal* I)	1641-1662
Fli-1 U1	5' GAGAGAAGCTTCGGGTCAATGTGTGGAATATTG 3' (*Hind* III)	197-218
SCF D1	5' TTAGGATCCACATGAACTGTTACCAGCC 3' (*Bam* HI)	1042-1060
SCF U1	5' CCGTCTAGACTCTTCTGGACAAGTTCTCAA 3' (*Xba* I)	432-452
c-kit D1	5' GAGAGAAAGCTTGGGGTCGGAGACAGCAGCAAA 3' (*Hind* III)	2980-3000
c-kit D2	5' GAGAGAAAGCTTCCAACCAGGAAAAGTTTGGCA 3' (*Hind* III)	373-393
c-kit U1	5' GAGAGACTGCAGCTCAGAGTCTAGCGCAGCCAC 3' (*Pst* I)	4-24
c-kit U2	5' GAGAGACTGCAGTATTTGCAGAAACCCATGTAT 3' (*Pst* I)	1664-1684
Spi-1 D1	5' GAGAGAGAATTCCTGGCGGTCTCTGCGGGCG 3' (*ECo* RI)	953-972
Spi-1 U1	5' GAGAGGTCGACCAACCTGGAGCTCAGCTGG 3' (*Sal* I)	132-151
Erg D1	5' CTTGGTCATGATGTTCTTGTC 3'	1331-1351
Erg U1	5' TCTCCACGGTTAATGCATGC 3'	927-946
EpoR D1	5' GGCCAGATCTTCTGCTGCAGAGTC 3' (*Pst* I)	853-875
EpoR U1	5' GTTTTGTGCCGCTGGAGCTGCAGG 3' (*Pst* I)	375-398

Numbering taken from the following references: *p53* (Bienz *et al*, 1984); *Fli-1* (Ben-David *et al*, 1991); *SCF* (Anderson *et al*, 1990); *c-kit* (Qiu *et al*, 1988); *Spi-1* (Klemsz *et al*, 1990); *Erg* (Rao *et al*, 1987); *epoR* (D'Andrea *et al*, 1989a).

For amplification of the non-coding region of *Fli-1*, 1μg of spleen DNA was used and the two primers shown in Table 6.3B.

All PCR reactions from genomic DNA were carried out under the same conditions used in the RT-PCR protocol (section 6.11). Products were purified on NACS columns prior to subsequent experimental manipulation.

6.13: Sequencing.

All sequencing was performed using the Sequenase Version 2.0 kit supplied by USB, according to the recommended protocol, incorporating [α-^{35}S] dATP as the radioactive label. However, to anneal the primer a slightly different approach from that recommended was found to give better results. 2μg of the template was boiled for 5min with 10ng of primer in 1x reaction buffer from the kit (40mM Tris.Cl (pH7.5), 20mM $MgCl_2$, 50mM NaCl), frozen on dry ice then slowly thawed to room temperature. The *erg*, *Fli-1*, *c-kit*, *SCF* and *Spi-1* plasmids were sequenced using either the primers used for the PCR, or the following two primers, available from USB, which anneal to region adjacent to the multiple cloning site of vectors used in this thesis:

M13 Reverse (-24): 5' AACAGCTATGACCATG 3'

M13 Universal: 5' TAAAACGACGGCCAGT 3'

For direct sequencing of *p53* PCR products, the same protocol was used with 0.5-1.5μg of the NACS column-purified DNA as a template. The following oligonucleotide, which hybridises to bases 557 to 577 of the *p53* cDNA (Bienz *et al*, 1984), was used to prime the sequence reaction in which the ELM deletion discovered: *p53* U3 5'-CTCCTCCCCAGCATCTTATCC-3'.

6.14: Electrophoretic mobility shift assays (EMSAs).

6.14.1: Preparation of probes.

Blunt-end 5'-end-labelled double-stranded oligonucleotides were prepared essentially as previously described (Plumb *et al*, 1989). 5μg of two complementary

TABLE 6.3.

Oligonucleotide primers for PCR from genomic DNA.

Name.	Sequence.	Position.
A. *p53*.		
For PCR across the ELM deletion:		
p53 U2	5' TCAGGCTTATGGAAACTGTGAGTGGAT 3'	67-83 (plus 10 nucleotides from intron 2)
p53 D2	5' CTTCCAGTGTGATGATGGTAAGGATAG 3'	740-766
To generate a *p53* intron 7 probe:		
p53 I75'	5' ATCATCACACTGGAAGACTCCAG 3'	751-773
p53 I73'	5' TGTCCCGTCCCAGAAGGTTCCCA 3'	774-796
To generate a *p53* intron 2 probe:		
p53 I25'	5' TCAGGCTTATGGAAACTGTGAGTGGAT 3'	67-83 (plus 10 nucleotides from intron 2)
p53 I23'	5' ATCTTCTGGAGGAAGTCTGGAAAACA 3'	84-99 (plus 10 nucleotides from intron 2)
B. *Fli-1*.		
To generate a probe for the non-coding region of the *Fli-1* gene:		
Fli-1 U2	5' TGAAGTCACTTCCCAAAATTAG 3'	5-26
Fli-1 D2	5' ACCCGGTTACAGCCTGACCTCG 3'	180-202

Numbering according to Bienz *et al*, 1984 (*p53*) and Ben-David *et al*, 1991 (*Fli-1*)

single-stranded oligonucleotides were annealed in 100µl of a 1x TE, 0.1M NaCl solution, by boiling for 10min in a water bath and then allowing to cool slowly to RTemp. 2µl from this solution was end-labelled with ^{32}P in the following reaction:

2µl of double-stranded oligonucleotide (100ng/µl)

0.5µl of 0.1M dithiothrietol

2µl of [γ-^{32}P] dATP

1µl of 10x kinase buffer (0.5M Tris.Cl (pH8), 0.1M $MgCl_2$, 5mM spermidine, 0.1mM EDTA, 1mM $ZnSO_4$)

3.5µl of distilled water

1µl T4 polynucleotide kinase (10U/µl)

which was incubated at 37°C for 30min, heated to 70°C for 5min, incubated for a further 10min at 37°C, then cooled to RTemp. The ragged ends of the oligonucleotide were filled in by adding to the end-label reaction mix, 2µl of a 5mM dNTPs solution, 6µl of water, 1µl of 10x TA (0.33M Tris.Ac (pH7.9), 0.66M KAc, 0.1M $MgAc_2$, 5mM dithiothrietol, 1mg/ml BSA) and 1µl of Klenow enzyme (1U/µl), and incubating on ice for 1hr. The oligonucleotide was then precipitated by adding 8µl of 1M NaCl, 15µg tRNA, 300µl of ethanol, cooling at -20°C for 2hrs, and then spinning for 15min in a microfuge. The pellet was resuspended in a 20% (v/v) glycerol solution and electrophoresed on a 1x TBE (90mM Tris, 90mM boric acid, 2mM EDTA), 8% (w/v) polyacrylamide gel at 100V for 90min in 1x TBE running buffer. The gel was exposed to Kodak X-OMAT AR imaging film for 10secs, the film developed, and the radiolabelled oligonucleotides excised from the gel by aligning the autoradiograph with the gel. A second exposure was then taken to ensure the oligonucleotide had been removed. The gel slice was then incubated at 37°C overnight in 1ml of 1xTE. To estimate the amount of unlabelled sequence used in competition experiments it was assumed that 50% of the oligonucleotide was lost during the procedure, giving a concentration of labelled oligonucleotide of 100ng/ml.

The sequence of the oligonucleotides used in EMSA experiments in this thesis are shown in Table 6.4.

TABLE 6.4.

Oligonucleotides for electrophoretic mobility shift assays.

Name.	Sequence (GGAA motif boxed).	Reference.
E74	GATCTCTAGCTGAATAACC[GGAA]GTAACTCATCCTAGGATC	Reddy and Rao, 1991.
E74(mut)	GATCTCTAGCTGAATAACC[CAA]GTAACTCATCCTAGGATC	Reddy and Rao, 1991.
PEA3	GATCCTCGAGCA[GGAA]GTTCGAGATC	Wasylyk *et al*, 1990.
PU-1	GATCCATAACCTCTGAAAGA[GGAA]CTTGGTTAGGTGATC	Klemsz *et al*, 1990.
α-P3a	GATCCAAACCAGCCAATGAGAACTGCTCCAGATC	Plumb *et al*, 1989.

6.14.2: Preparation of nuclear proteins from mammalian cell lines.

Nuclear proteins were extracted using a rapid extraction protocol as follows. $5x10^7$ cells were homogenised in hypotonic buffer (10mM Tris.HCl pH 7.4, 10mM NaCl, 1.6mM $MgCl_2$, 1mM $CaCl_2$) then spun at 2,000rpm in an IEC Centra-8R centrifuge at 4°C for 10min. The nuclear pellet was washed with 10ml of TMS (0.25M sucrose, 5mM $MgCl_2$, 10mM Tris.HCl pH 7.4) then resuspended gently in 100µl of storage buffer (50mM NaCl, 20mM Hepes pH 7.9, 5mM $MgCl_2$, 0.1mM EDTA, 1mM DTT and 20% glycerol). One tenth volume of 4M NaCl was added, mixed and incubated on ice for 20min, then spun at 55,000rpm at 4°C for 1hr in a Beckman Benchtop Ultra-Centrifuge and the supernatant stored at -70°C.

The relative concentration of proteins in the samples was assessed by comparing αP3a binding activity in 2 and 5µl aliquots. This sequence contains the CCAAT box of the mouse α-globin promoter which is thought to bind to a ubiquitous protein present at similar amounts in many cell types (Plumb *et al*, 1989; Cohen *et al*, 1986). It seemed unlikely that their would be any gross differences in the expression of this protein between the various cell lines of the ELM system. In subsequent EMSAs, the volume of nuclear protein used was adjusted to compensate for differences in the αP3a activity.

6.14.3: DNA:protein binding reaction and gel electrophoresis.

The appropriate volume of protein extract (1 to 5µl) was incubated for 10min on ice with 6µg of poly (dI-dC) and 5 to 500ng of any appropriate unlabelled competitor oligonucleotides, made up to a final volume of 15µl with storage buffer. ~500pg of labelled oligonucleotide was added to each sample, made up to 20µl with storage buffer, and incubated for 20min at room temperature. Samples were electrophoresed in a 0.25x TBE, 5% (w/v) polyacrylamide gel in 0.25x TBE running buffer (pre-run for 1hr at 150V) for 2hrs at 150V at 4°C; the gel was then dried for autoradiography.

6.15: *In vitro* transcription-translation.

1µg of the p*Fli* vector was linearised with *Sal* I then incubated in 1x transcription buffer (40mM Tris.Cl (pH7.5), 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl) with 2.5mM dNTPs, 10mM dithiothreitol, 40U of RNasin and 30U of T7 RNA polymerase in a final volume of 20µl for 90min at 37°C. 2µl from this transcription reaction was then used in the translation reaction, performed in a nuclease-treated rabbit reticulocyte lysate (purchased from Promega) according to the manufacturers instructions. Aliquots from this reaction were then used in EMSAs as described above. A control reaction was performed in which the p*Fli* vector was omitted.

6.16: Isolation of lambda 'phage clones.

6.16.1: Preparation of plating cells.

E. coli strain P2392 were used throughout the preparation and screening of the genomic library (section 6.3.1). To make the cells competent for 'phage infection, 50ml of NZCYM medium containing 0.2% (w/v) maltose was inoculated with a single colony of P2392 cells from an NZCYM agar plate and grown at 37°C overnight. This induces expression of the maltose receptor which also acts as a receptor for bacteriophages. The cells were pelleted by centrifugation at 3,000 rpm for 25min in an IEC Centra-8R centrifuge, resuspended in 20ml of SM (0.1M NaCl, 5mM $MgSO_4$, 50mM Tris.Cl (pH7.5), 0.1% (w/v) gelatine) and stored at 4°C until required. Cells were prepared fresh for each infection.

6.16.2: Plating 'phage.

To titre and amplify 'phage stocks, 400µl of plating cells were incubated with 10µl of various dilutions of the 'phage stock in a Falcon 2059 tube at 37°C for 20min. 6.5ml of molten 0.6% (w/v) agarose NZM (45°C) was added to the tube and the mix poured onto a 150mm diameter plate containing 1.5% (w/v) agar NZM. This was allowed to solidify for 15min and then incubated inverted overnight at 37°C. To plate

'phage for screening, large 200mm^2 plates were employed with the incubation mixes scaled up appropriately.

6.16.3: Preparation of a genomic library from ELM-I-1 DNA.

10µg aliquots of I-1 genomic DNA were digested with a range of *Sau* 3AI concentrations to optimise reaction conditions such that the majority of the subsequent DNA fragments were between 12 and 20kb. The reaction was then scaled up to generate 12-20kb fragments from 300µg of DNA: these fragments were isolated by electrophoresis on a 0.4% (w/v) low melting point agarose gel followed by electroelution and NACS column purification (section 6.5.3), and finally resuspended to an approximate concentration of 0.5mg/ml. The vector used in forming the library was λGEM11, digested with *Bam* HI and *ECo* RI, purified on a NACS column and resuspended to a final concentration of 0.5mg/ml. 0.125-1µg of the *Sau* 3AI partially-digested DNA was ligated into 0.5µg of *Bam* HI/*ECo* RI λGEM11 using T4 DNA ligase (section 6.5.4). One fifth of this reaction was packaged using Gigapack Plus packaging extracts, according to the manufacturers instructions, and suspended to a final volume of 100µl with SM. 10µl of chloroform was added, the mixture vortexed, the two layers were allowed to separate and the stock stored at 4°C. The chloroform prevents bacterial contamination of the 'phage solution. 1µl was plated to assess the number of plaque-forming units (pfu) present in the stock. If the titre was greater than 250,000 pfu/ml, then the remainder of the ligation mixes were packaged.

6.16.4: Amplification of the library.

Fifteen 150mm diameter dishes, each containing 100,000 pfu of the library, were covered with 12ml of SM and incubated overnight at 4°C. The SM was then spun at 3,000rpm for 15min in an IEC Centra-8R rotor to remove agarose fragments, and the supernatant then transferred to sterile 20ml glass tubes, 200µl of chloroform added, vortexed and stored at 4°C. The amplified library was titred as described above.

6.16.5: Screening the library with radiolabelled DNA probes.

~150,000 pfu of the amplified or unamplified library were plated onto 200mm^2 NZM plates as described above (section 6.16.2) and incubated overnight at 37°C. The plates were then cooled at 4°C for 2hrs. Duplicate lifts were taken off each plate onto nitrocellulose Hybond-C membranes purchased from Amersham, and subsequently denatured and neutralised according to their instructions. DNA was fixed to the filter by baking in an 80°C vacuum oven for 2hrs. The filters were prehybridised, hybridised to radiolabelled DNA probes, washed and exposed in an identical fashion to the Southern blots (section 6.9). A Southern blot of DNA from mouse spleen was included in these procedures to verify the quality of the probe being used. Putative positive plaques were cut from the original plate, incubated in 1ml of SM, plus 50µl of chloroform, at 4°C overnight, titred, then rescreened with the same probe. Single positive plaques from this secondary screen were screened for a third time to verify their identity. Single plaques from the tertiary screen were used to prepare DNA for further analysis.

Three probes from the *Fli-1* locus were used to screen the library: SU-9 (see table 6.1), the full-length *Fli-1* cDNA, and a PCR product generated from the 5' non-coding region of the *Fli-1* gene (section 6.12). The latter probe proved to be difficult to use due to its short length (<200bp) reducing hybridisation efficiency, and the two clones described in detail in this thesis were isolated using the SU-9 probe.

6.16.6: Preparation of 'phage DNA.

1ml of fresh P2392 plating cells were incubated in a Falcon 2059 tube with 10^7 pfu from a stock of the positive recombinant 'phage, incubated for 20min at 37°C and then used to inoculate 500ml of NZCYM. After incubation for between 12-16hrs the cells lyse, apparent from the accumulation of cell debris in the culture. 10ml of chloroform was then added and the mixture shaken at 225rpm for 15min in a New Brunswick G25 shaker. The lysate was then cleared by centrifugation at 3,000rpm for 15min in an IEC Centra-8R rotor. The DNA was isolated using the glycerol step

gradient method, after first precipitating the 'phage particles using polyethylene glycol (8000) (described in detail in Sambrook *et al*, 1989).

6.16.7: Restriction mapping and characterisation of 'phage clones.

The approximate length of the genomic DNA insert present in positive 'phage clones was determined by cleavage with *Sfi* I and subsequent electrophoresis on a 0.8% (w/v) low melting point agarose gel. ~10μg of the insert was then isolated by *Sfi* I digestion, electrophoresis, and NACS purification (section 6.5.3). A series of Southern blots were then prepared (section 6.9) in which 0.5μg of this *Sfi* I fragment had been cleaved with a second enzyme. The probes hybridised to these blots were prepared by transcription labelling from the T7 and SP6 promoters in the λGEM11 vector, performed as follows. 10μg of the clone DNA was cleaved with *Hae* III, ϕOH/$CHCl_3$ extracted, ethanol precipitated and resuspended in 10μl of DEPC-treated water. 1μl of this DNA preparation was then incubated in 1x transcription buffer (section 6.15) with 1μl of rATP, rGTP and rCTP (all 10mM), 1μl 0.5M dithiothrietol, 20U RNasin, 5μl [α-^{32}P] rUTP and 10U of T7 or SP6 RNA polymerase in a final volume of 25μl. The reaction was incubated at 37°C for 30min and the labelled fragments separated from unincorporated nucleotides by gel filtration on a Sephadex G-50 column (section 6.10). The probe was hybridised to the filters after heating to 65°C for 5min using the same procedure used for Northern blot analysis (section 6.8).

Filters were stripped and hybridised with probes from the *Fli-1* locus, such as SU-9 and the 5' non-coding region of the cDNA, to further map restriction sites. Once a comprehensive map had been generated, fragments of interest were cloned into pBluescriptII KS+ and sequenced, as described above. Comparisons were made with known sequences using the VAX (Devereux *et al*, 1984).

6.17: Immunostaining ELM-D cells.

ELM-D cells growing on MS-5 stromal cells were seeded at 10% confluence in a sterile 100mm diameter Petri dish containing 10ml of ELM growth medium (section

6.1.1) and a number of glass coverslips. Cells were grown for three days until ~75% confluence; the medium was then removed and the cells washed three times with PBS. After a brief rinse in fixing solution (50:50 (v/v) mix of methanol and acetic acid) cooled to -20°C, cells were left in fresh fixing solution for 10min at -20°C. After a brief rinse with cold PBS, individual coverslips were then used in the immunostaining protocol, which used the Vectastain ABC-AP kit (mouse IgG) and the anti-p53 antibody PAb 246 as the primary antibody (gift from Dr. D. Lane; Yewdell *et al*, 1986). The fixed cells were treated according to the method recommended by the manufacturers of the kit with the help of M. Clarke. The protocol employed a biotinylated horse anti-mouse IgG antibody to detect PAb 246:p53 complexes, which is then itself bound to an avidin molecule linked to horseradish peroxidase. Then, by incubating with a peroxidase substrate (diamino benzidine), a brown stain is formed coincident with the position of the PAb246:p53 complexes. The stained cells were dehydrated by washing with increasing concentrations of ethanol, up to 100%, air dried, mounted, and photographed with a Leitz microscope set at a range of magnifications.

As a control, an identical procedure was followed using a separate coverslip of cells, except that the PAb 246 binding step was omitted.

CHAPTER 7: Materials.

7.1: Murine cell lines, media and tissue culture supplies.

The MS-5 stromal feeder cell line, and all the ELM cell lines and tumour cells, were obtained from Dr. W. Ostertag. F4-12 cells were taken from laboratory stocks.

Supplier: Beatson Institute Central Services.

Penicillin (7.5mg/ml).

Streptomycin (10mg/ml).

Sterile CT buffer.

Sterile PE.

Sterile PBS.

Sterile glassware and pipettes.

Supplier: Fisons Scientific Equipment, Loughborough, Liecs., England.

DMSO.

Supplier: Gibco Europe Life Technologies Ltd., Paisley, Scotland.

α-Minimal Essential Medium, plus deoxyribonucleosides and ribonucleosides.

Ham's F12 Medium.

Special Liquid Medium.

Foetal Calf Serum.

2.5% (w/v) Trypsin.

200mM Glutamine.

Supplier: Sigma Chemical Co. Ltd., Poole, Dorset, England.

Donor Horse Serum.

Supplier: A/S Nunc, Roskilde, Denmark.

Tissue culture flasks.

Nunc tubes.

Supplier: Becton Dickinson Labware, Plymouth, England.

100mm diameter tissue culture dish.

Recombinant human erythropoietin was the generous gift of Dr. T. Holyoake, whilst IL-3 was purified from WEHI-3 conditioned-medium by Dr. J. Ihle. These were used in differentiation induction experiments at 2U/ml and 10U/ml, respectively.

7.2: Bacterial hosts and media.

E. coli host strain JM83 was obtained from laboratory stocks held by M. Walker. DH5α competent cells were purchased from Gibco Europe Life Technologies Ltd., Paisley, Scotland. *INVαF'* cells came with the TA cloning kit (section 7.6), whilst P2392 cells used for 'phage propagation were the generous gift of Dr. C. Bartholomew.

Terrific broth, NZCYM and NZM were prepared according to Sambrook *et al* (1989). Components of the media were purchased from BDH Chemicals Ltd., Poole, Dorset, England, unless they are included in the following list:

Supplier: Beatson Institute Central Services.

L-broth (prepared according to Sambrook *et al* (1989)).

Kanamycin (10mg/ml)

Sterile glassware.

Supplier: Difco, Detroit, Michigan, USA.

Agar.

Bacto-tryptone.

Casamino Acids.

Supplier: Sigma Chemical Co. Ltd., Poole, Dorset, England.

Ampicillin.

X-gal.

Supplier: Gibco Europe Life Technologies Ltd., Paisley, Scotland.

NZ broth.

Supplier: Beta Laboratories, East Molesey, Surrey, England.

Yeast extract.

7.3: Plasticware.

Supplier: Becton Dickinson Labware, Plymouth, England.

Falcon tubes.

Supplier: Bibby-Sterilin Ltd., Stone, Staffs., England.

All bacteriological dishes.

30ml Universal tubes.

7.4: Mice.

Female C3H mice were purchased from Harlem-Olac and housed by S. Bell. Injection, sacrifice and dissection were performed with the help of T. Hamilton.

Phenylhydrazine, used to induce anaemia, was purchased from Sigma Chemical Co. Ltd., Poole, Dorset, England.

The Beatson Institute's Alycon II Teletherapy Unit was used for all irradiation procedures.

7.5: Plasmids and bacteriophages.

pBluescriptII KS+ was supplied by Stratagene Ltd., Cambridge, England, and pUC19 by Gibco Europe Life Technologies Ltd., Paisley, Scotland, and used to propagate a number of DNA fragments generated during this thesis. Recombinant plasmids supplied by other workers are shown in Table 6.1. The λGEM11 'phage vector was the generous gift of Dr. C. Bartholomew.

7.6: Kits.

Supplier: Invitrogen Corporation, San Diego, California, USA.

TA Cloning kit.

Supplier: Bio 101 Inc., Stratatech Scientific, Luton, England.

Geneclean kit.

Supplier: Boehringer Mannheim UK, Lewes, East Sussex, England.

Random-Primed DNA Labelling kit.

Supplier: United States Biochemical, Cleveland, Ohio, USA.

Sequenase Version 2.0 kit.

Supplier: Promega, Madison, Wisconsin, USA.

Nuclease-treated rabbit reticulocyte lysate *in vitro* translation system.

Supplier: Stratagene Ltd., Cambridge, England.

Gigapack Plus bacteriophage packaging extracts.

Supplier: Vector Laboratories, Burlingame, California, USA.

Vectastain ABC-AP kit (mouse IgG).

7.7: Membranes, paper and X-ray film.

Supplier: Amersham International plc, Amersham, Bucks., England.

Hybond N nylon membranes.

Hybond C nitrocellulose membranes.

Supplier: Bethesda Research Laboratories, Gibco Ltd., Paisley, Scotland.

Dialysis tubing.

Supplier: Vernon-Carus Ltd., Preston, Lancs., England.

Gauze swabs.

Supplier: Whatman International Ltd., Maidstone, Kent, England.

3MM filter paper.

Supplier: Eastman Kodak Co., Rochester, New York, USA.

X-ray film (X-OMAT AR).

Duplicating film (DUP-1).

Supplier: Presentation Technology Ltd., Clydebank, Scotland.

AGFA Rapitone paper (P1-2 and P1-4).

7.8: Nucleotides, polynucleotides, RNA and DNA.

The following nucleotides, used to radiolabel DNA or RNA fragments, were purchased from Amersham International plc, Amersham, Bucks., England:

[α-^{32}P] dCTP ~3,000 Ci/mmol.

[α-^{35}S] dATP ~1,000 Ci/mmol.

[α-^{32}P] rUTP ~800 Ci/mmol.

[γ-^{32}P] dATP ~5,000 Ci/mmol.

Unlabelled nucleotides were also supplied by Amersham.

Supplier: Pharmacia Ltd., Milton Keynes, Bucks, England.

Poly (dI-dC).

Supplier: Sigma Chemical Co. Ltd., Poole, Dorset, England.

E. coli tRNA.

Herring testis DNA.

Supplier: Promega, Madison, Wisconsin, USA.

Oligo-dT 15mer.

Total cellular RNA from the Jurkat T cell line was the generous gift of Dr. R. Fulton.

Murine kidney RNA was the generous gift of Dr. C. Bartholomew.

7.9: Gels and columns.

Supplier: Bethesda Research Laboratories, Gibco Ltd., Paisley, Scotland.

Agarose and low melting-point agarose (Ultrapure grade).

NACS Prepac columns.

DNA markers (1mg/ml): 1kb ladder, bacteriophage ϕx174 DNA (*Hae* III-cut) and bacteriophage λ DNA (*Hind* III-cut).

0.24-9.5kb RNA ladder (1mg/ml).

Supplier: Severn Biotech Ltd., Kidderminster, England.

Design-a-Gel 40% (w/v) acrylamide: 2% (w/v) bis-acrylamide solution.

Supplier: Pharmacia Ltd., Milton Keynes, Bucks, England.

Sephadex G-50.

Supplier: Bio-Rad Laboratories, Richmond, California, USA.

Bio-Rad Bio Gel A-50M.

7.10: Enzymes and enzyme inhibitors.

Supplier: Bethesda Research Laboratories, Gibco Ltd., Paisley, Scotland.

All restriction endonucleases with buffer concentrates, with the exception of *Dra* III.

T4 DNA ligase (1U/μl) and 5x ligation buffer.

Proteinase K.

Taq DNA polymerase (10U/μl) and 10x PCR reaction buffer.

SP6 (15U/μl) and T7 (50U/μl) RNA polymerases and 10x transcription buffer.

Supplier: Boehringer Mannheim UK, Lewes, East Sussex, England.

Dra III (10U/μl) and 10x buffer.

Calf intestinal alkaline phosphatase (1U/µl).

DNAse-free RNAse.

RNAse A.

Supplier: Northumbria Biologicals Ltd., Cramlington, Northumberland, England.

T4 polynucleotide kinase (10U/µl).

Klenow DNA polymerase (1U/µl).

Supplier: Promega, Madison, Wisconsin, USA.

M-MLV Reverse transcriptase (200U/µl) and 5x H-RT buffer.

RNAsin (20U/µl).

Supplier: Sigma Chemical Co. Ltd., Poole, Dorset, England.

Diethylpyrocarbonate (DEPC).

Lysozyme.

Protease inhibitors, used to prepare nuclear protein extracts, were the generous gift from J. O'Prey.

7.11: Chemicals.

All other chemicals not listed above, were obtained (AnalaR grade) from BDH Chemicals Ltd., Poole, Dorset, England, with the following exceptions:

Supplier: Sigma Chemical Co. Ltd., Poole, Dorset, England.

Bromophenol Blue.	RbCl.
Dithiothreitol.	Spermidine.
Methylene Blue.	TEMED.
MOPS.	Triton X-100.
PIPES.	Xylene Cyanol.
Polyvinylpyrollidone.	$ZnSO_4$.

Supplier: James Burrough Ltd., Witham, Essex, England.

Ethanol.

Supplier: Rathburn Chemicals Ltd., Walkerburn, Scotland.

Water-saturated phenol.

Supplier: Pharmacia Ltd., Milton Keynes, Bucks, England.

Ficoll 400.

Supplier: Cinna/Biotecx Laboratories Inc., Houston, Texas, USA.

RNAzol B.

Supplier: Northumbria Biologicals Ltd., Cramlington, Northumberland, England.

Bovine Serum Albumin (20% w/v).

Supplier: Fisons Scientific Equipment, Loughborough, England.

Formaldehyde (38% w/v).

Supplier: Fluka Chemika-Biochemika AG, Buchs, Switzerland.

Formamide.

7.12: Water.

Distilled water for buffers and general solutions was obtained from a Millipore MilliRO 15 system. Water for protein/enzyme work or recombinant DNA protocols was further purified on a Millipore MilliQ system to 18MΩcm.

PART 3: RESULTS.

CHAPTER 8: Tumorigenicity and Differentiation differences within the ELM system.

8.1: Relative tumorigenicity.

The original tumour can be passaged *in vivo*, and it has been shown that both ELM-D and I-1 are tumorigenic (Itoh *et al*, 1988). To compare tumorigenicity within the system, cells were injected via the tail vein into syngeneic C3H mice that had been immunosuppressed by irradiation with 300 Rads of γ-rays. Initially, four of the stroma-independent cell lines (I-1, I-2, I-5, I-6) were tested. For each cell line, 10^7 cells were injected into five mice which were sacrificed 15 days post-injection, at which time their spleens were weighed as a measure of leukaemogenicity (Figure 8.1, graph A). Those injected with I-1 and I-5 showed a significant splenomegaly, up to eight times that of a normal spleen, often accompanied by enlargement of the liver (indicated by the letter 'L'), whilst I-2 and I-6 showed only a moderate increase in spleen weight with the liver remaining normal. Although I-5 induces a more rapid splenomegaly than I-1, mice injected with I-1 cells appeared much less healthy prior to sacrifice. This is probably a consequence of severe anaemia since the I-1 spleens were significantly paler than those taken from other animals.

The stromal cell-independent cell lines were then compared with D#6 cells, which were grown on lethally-irradiated MS-5 feeders to remove any influence they may have on tumorigenicity. The same procedure was used except that fewer cells had to be used as the D#6 cells tended to clump with the feeders at high concentrations and resulted in several mice dying shortly after injection, probably as a result of blood vessel blockage. Prior to harvesting the cells from tissue culture flasks, it was estimated visually that the D#6 to MS-5 cell ratio was 6 to 1: the appropriate adjustment was made so that equal numbers of D#6 and stromal cell-independent cells were used in subsequent experiments. Two groups of immunosuppressed mice received $2x10^6$ or $6x10^6$ cells and were sacrificed after 20 days and 15 days respectively. The spleen weights confirmed the differences between

Figure 8.1: Relative tumorigenicity within the ELM system.

Cells were harvested and the specified number injected into immunosuppressed C3H mice as described in the Methods and Materials. After an appropriate incubation period when the animals began to appear unhealthy, the mice were sacrificed and their spleens weighed. Each bar is the average spleen weight from five injected mice, with the standard deviation shown by the error bar. The letter 'L' represents groups in which all the animals also exhibited significant hepatomegaly.

Graph **A**: 10^7 cells injected; incubation period of 15 days.
Graph **B**: $2x10^6$ cells injected; incubation period of 15 or 20 days as indicated.
Graph **C**: $6x10^6$ cells injected; incubation period of 15 days.

Cells tested were:
I-1, I-2, I-5 and **I-6**:- Stromal cell-independent clones I-1, I-2, I-5 and I-6.
D#6:- ELM-D#6 cells growing on lethally-irradiated MS-5 cells (6 D#6 cells per MS-5).
IVP ELM:- *In vivo* passaged ELM tumour cells.
M:- Lethally-irradiated MS-5 cells (NB: Only one seventh the number of cells indicated were injected to control for MS-5 cells included in the D#6 injection).

'**N**' shows the average weight of the spleen from an untreated C3H mouse of the same age and sex as those used in the experiment.

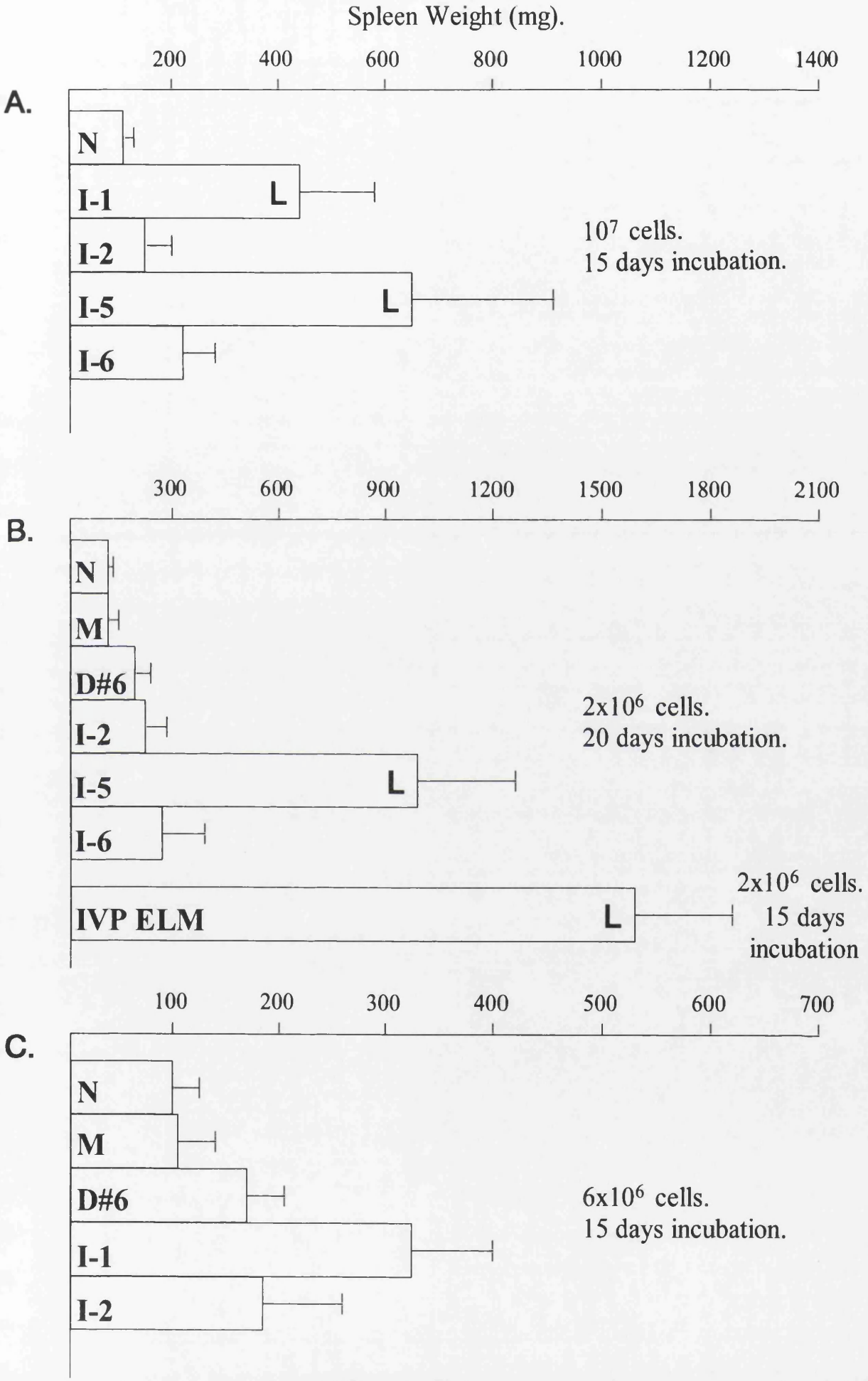

Spleen Weight (mg).
200
400
600
800
1000
1200
1400
A.
N
I-1
L
I-2
I-5
L
I-6
10^7 cells.
15 days incubation.
300
600
900
1200
1500
1800
2100
B.
N
M
D#6
I-2
I-5
L
I-6
IVP ELM
L
2x10^6 cells.
15 days
incubation
2x10^6 cells.
20 days incubation.
100
200
300
400
500
600
700
C.
N
M
D#6
I-1
I-2
6x10^6 cells.
15 days incubation.

Figure 8.1.

the stroma-independent cell lines, whereas the mice injected with D#6 showed a weight increase similar to that seen with I-2 (Figure 8.1, graphs B and C). As a control, other mice received irradiated MS-5 cells only. Six-fold fewer cells were used to give an amount roughly equivalent to the number of MS-5 cells injected along with D#6. These animals showed no significant splenomegaly after the specified incubation period.

In a parallel experiment, $2x10^6$ spleen cells that had been passaged *in vivo* from the original erythroleukaemia were similarly injected into irradiated recipient mice. Again a group of 5 mice was used which had to be sacrificed only 15 days post-injection due to their unhealthy appearance. The mice were not anaemic, but their spleens were enormous, on average sixteen times larger than normal mice (Figure 8.1, graph B). All contained a liver at least twice the normal size. The rapid enlargement of these organs engorged with differentiating leukaemic cells may arise for a number of reasons. First, it may simply reflect a technical problem: it is more difficult to accurately estimate the number of leukaemic cells in a homogenised spleen preparation, than cells harvested from a tissue culture flask, a problem that could have resulted in the injection of the inappropriate number of cells. Nevertheless, it seems unlikely that this fully explains the rapid splenomegaly observed in mice receiving *in vivo* passaged tumour cells. A second possibility is that normal haemopoietic and stromal cells, injected in combination with the erythroleukaemic cells, enhance their proliferative capacity or viability. Alternatively, continuous passage may have selected for clones with a greater proliferative capacity than the cells that gave rise to the ELM cell lines. This does not necessarily have to be due to a genetic change, but may simply reflect epigenetic conditioning for growth *in vivo*, which is absent from the cells cultured *in vitro* that will not have been subjected to the same selective pressures. Re-injection of leukaemic spleen cells arising in mice injected with the cell lines, may induce tumours more rapidly than when freshly-harvested cells from tissue culture are used, but this has yet to be tested. However, despite this possible selection, the *in vivo*

passaged tumour cells seem not to have lost their stromal cell-dependence as they still appear to require stromal cells for long-term viability *in vitro*: seeding cells from a homogenised ELM spleen in ELM growth medium (see Methods) results in a rapid reduction in cell numbers which is followed by the formation of small colonies of ELM cells on stromal cells present in the spleen cell homogenate. Inclusion of additional stromal cells, in the form of a MS-5 cell layer, reduces the initial loss of cell viability (data not shown). Whatever the explanation, this result does not detract from the obvious differences that exist between the ELM cell lines with respect to both growth and differentiation *in vivo*.

8.2: Differentiation induction *in vitro* in response to epo and IL-3.

Since mice injected with I-1 appeared more anaemic than mice injected with other ELM cell lines, their differentiation capacity was tested *in vitro* in response to erythropoietin (epo) and interleukin-3 (IL-3), physiological inducers of erythropoiesis. Cells were grown to near confluence, harvested and reseeded at $2.5x10^5$ cells/ml in assay medium containing 2U/ml of epo and/or 10U/ml of IL-3 (see Methods and Materials). After 72 hours, the cells were harvested and, as expected from the *in vivo* observations, all the ELM cell lines examined, with the exception of I-1, had developed a red colour. To quantify this effect, RNA was extracted and analysed by Northern blotting (Figure 8.2). Probing with a mouse α-*globin* genomic fragment (spanning exons 1 and 2 of the gene) demonstrated that I-1 differentiated only slightly in response to epo and undetectably in IL-3, whilst the other independent cell lines exhibited extensive α-*globin* mRNA induction with epo, an effect slightly enhanced by the presence of IL-3. F4 erythroleukaemic cells, which arose from SFFV infection (Ostertag *et al*, 1972), show the same insensitivity to epo and IL-3 as that seen in the I-1 line. The blot was stripped of the radiolabelled α-*globin* probe and hybridised to a cDNA fragment from the *GAPDH* gene to quantitate the amounts of RNA loaded in each of the lanes. The D#6 loading appears significantly higher than the other samples, but this is simply because it is a longer

Figure 8.2: Differentiation capability of erythroleukaemia cell lines in response to erythropoietin and/or interleukin-3.

Freshly-harvested cells were incubated for three days in assay medium (see Methods and Materials) containing either no additional growth factors or erythropoietin (2U/ml) and/or interleukin-3 (10U/ml), indicated by the '+' and '-' symbols. RNA was extracted and 20μg used to prepare a Northern blot which was hybridised to a ^{32}P-radiolabelled α-*globin* probe as a marker of erythroid differentiation. Exposure time: 3 hours. The blot was stripped of the α-*globin* probe and hybridised to a *GAPDH* cDNA fragment to assess RNA loading. Exposure time: 12 hours.

Cell lines tested were:
I-1, I-2, I-5 and **I-6**:- Stromal cell-independent clones I-1, I-2, I-5 and I-6.
D#6:- ELM-D#6 cells growing on live MS-5 cells (6 D#6 cells per MS-5).
F4:- Friend virus-induced erythroleukaemia cell line, F4-12.

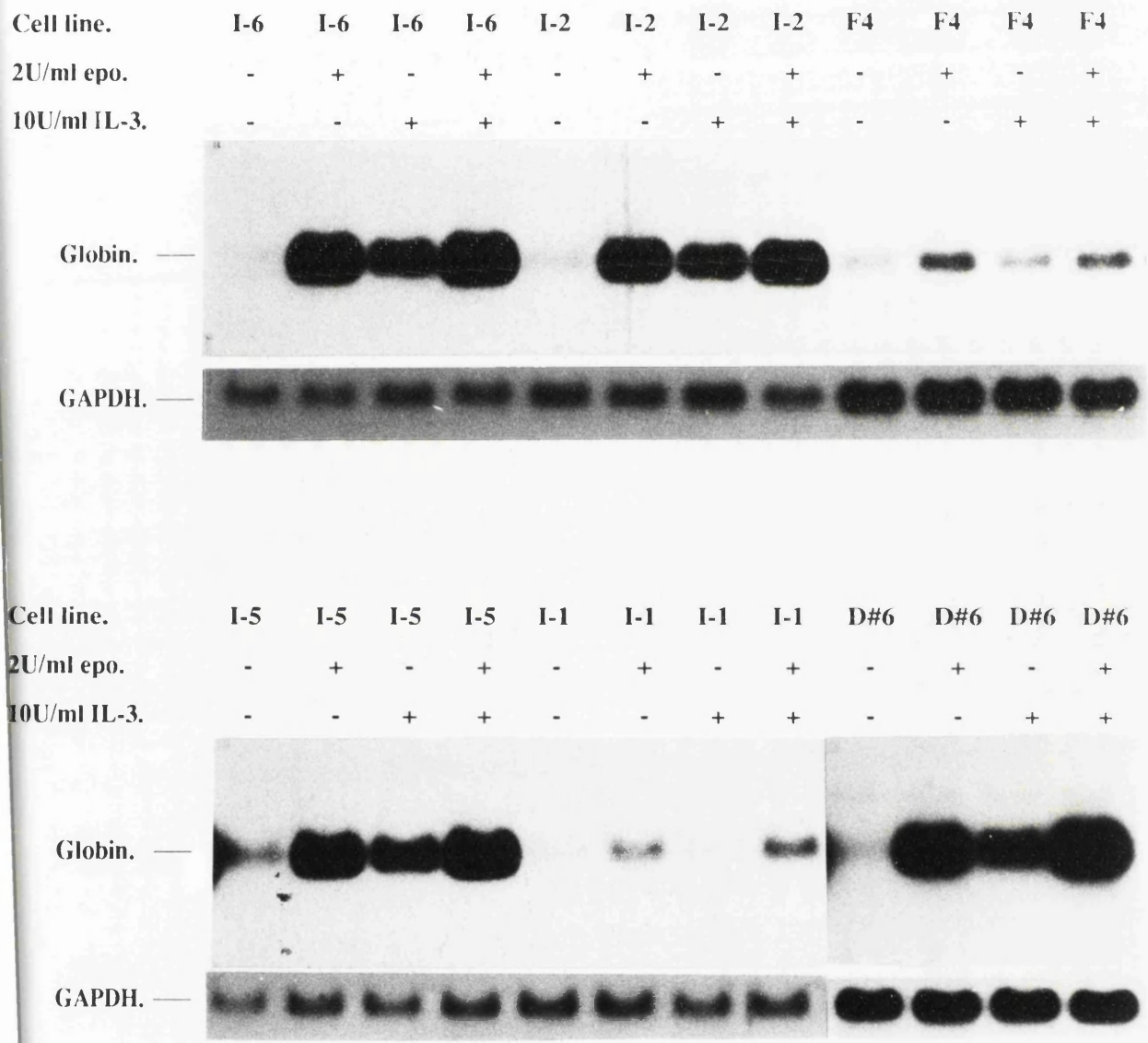

Cell line. I-6 I-6 I-6 I-6 I-2 I-2 I-2 I-2 F4 F4 F4 F4
2U/ml epo. - + - + - + - + - + - +
10U/ml IL-3. - - + + - - + + - - + +
Globin.
GAPDH.
Cell line. I-5 I-5 I-5 I-5 I-1 I-1 I-1 I-1 D#6 D#6 D#6 D#6
2U/ml epo. - + - + - + - + - + - +
10U/ml IL-3. - - + + - - + + - - + +
Globin.
GAPDH.

Figure 8.2.

exposure of a separate blot: when this is taken into account, the extent of α-*globin* expression is only slightly lower in the D#6 cells, probably the result of contaminating stromal cells (data not shown). Identical results to those presented here were seen after 5 and 7 day inductions with epo and/or IL-3 (data not shown).

Surprisingly, α-*globin* gene expression and haemoglobin production were enhanced by treatment with IL-3 alone. Inspection of the pellet of harvested cells suggested that this was confined to a small population of differentiating cells, coloured red by haemoglobin production. One possible explanation for this result is that IL-3, conventionally viewed as an enhancer of erythroid progenitor growth/survival not differentiation, synergises with low concentrations of epo in the serum allowing more cells to become committed to differentiation.

The insensitivity of I-1 cells to epo, appears not to be due to the level of *epo receptor* (*epoR*) gene expression (Figure 8.3). Northern blot analysis, using an *epoR*-specific cDNA probe generated by RT-PCR, has shown that, once the relative loading of the RNA samples has been taken into account, *epoR* gene expression is at a similar level in all the ELM cell lines, although possibly slightly lower in I-1 cells (Figure 8.3B). Furthermore, Shiozaki *et al* (1990b) have demonstrated that I-1 cells possess epo binding sites on their cell surface. They also showed that on exposure to epo, I-1 cells exhibited an increased proliferation, and a slight differentiation induction (similar to that shown in Figure 8.2). Thus, I-1 cells are probably able to generate intracellular signals through epoR as a result of ligand binding, but their ability to respond by differentiating is more efficiently blocked than other ELM cell lines. One possibility which this experiment does not address, is that different forms of the epo receptor, like the truncated receptor created by alternative-splicing of nascent mRNA (Nakamura *et al*, 1992), may be present on the various cell lines which may alter the cellular effect exerted by ligand binding.

One interesting, though surprising, observation is the detection of *epoR* expression in the MS-5 stromal cells (Figure 8.3), a result confirmed by Ostertag and his colleagues (personal communication). The significance of this is unclear, but it is

Figure 8.3: Expression of the *erythropoietin receptor* (*epoR*) gene in the ELM system.

A: Position of PCR primers (***epoR* U1** and ***epoR* D1**) used to generate a cDNA probe for *epoR* by RT-PCR (see Methods and Materials). The identity of the product was confirmed by cloning the fragment into pBluescriptII KS+ followed by partial sequencing of the termini of the insert. Primer sequences are shown in Table 6.2 in the Methods and Materials section. The structure of the cDNA is from D'Andrea *et al*, 1989a. The position of the region encoding the putative membrane spanning region of the protein is indicated by 'TM'.

B: Northern blot of 20μg samples of total cellular RNA hybridised to the *epoR* cDNA fragment labelled with ^{32}P (Exposure time: 3 days), stripped and then hybridised to a similarly labelled *actin* cDNA to assess RNA loading (Exposure time: 12 hours). Molecular weight markers are given in kb to the right of the blot.

Cell lines and tissues tested were:
I-1, **I-2**, **I-5** and **I-6**:- Stromal cell-independent clones I-1, I-2, I-5 and I-6.
MS-5:- Stromal cell line, MS-5.
ELM-D:- Uncloned ELM-D cells growing on live MS-5 cells (7 ELM-D cells per MS-5).
IVP ELM:- *In vivo* passaged ELM tumour cells.
Spleen and **kidney** samples from a C3H mouse.
F4-12:- Friend virus-induced erythroleukaemia cell line, F4-12.

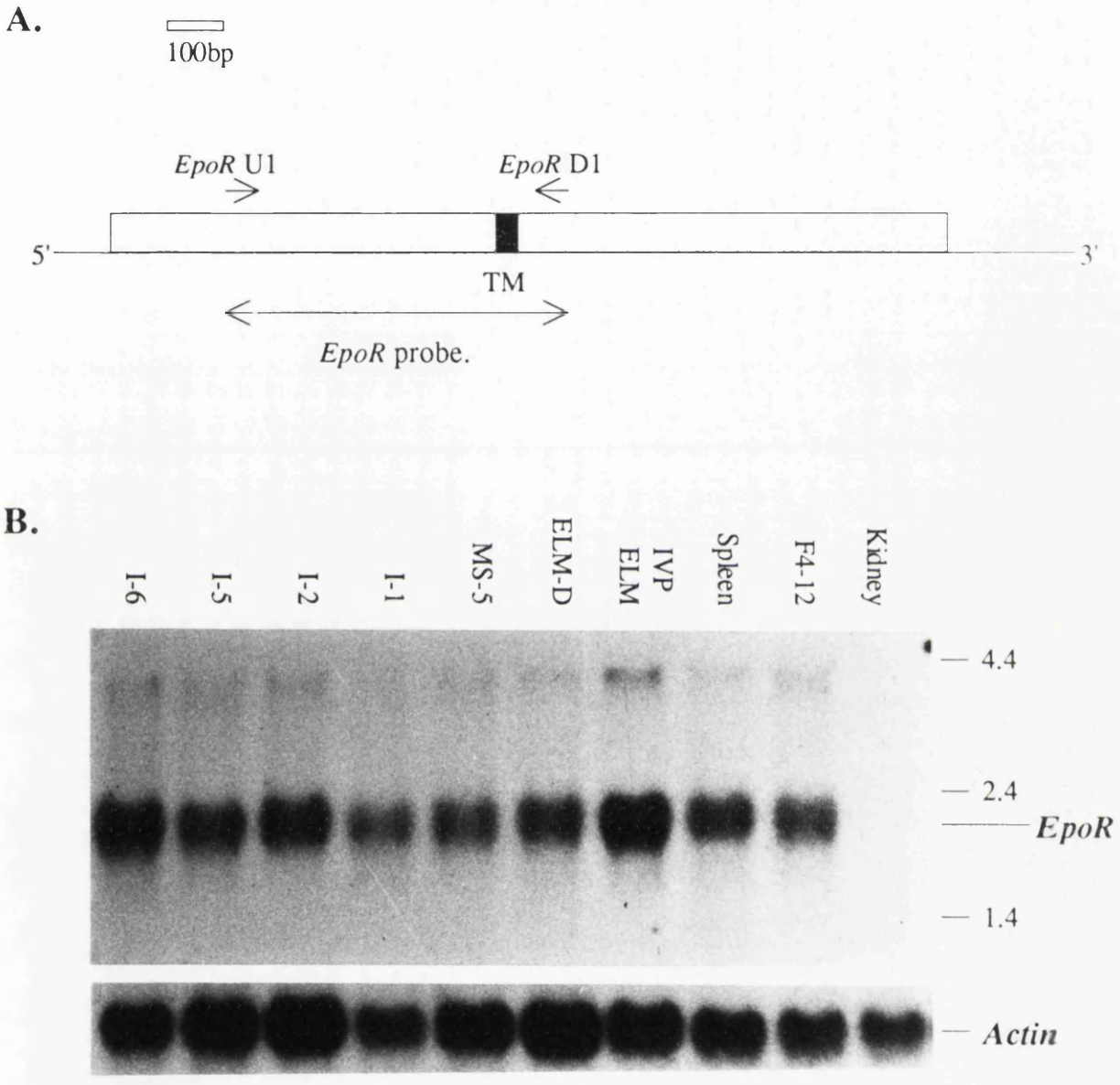

A.
100bp
EpoR U1
EpoR D1
5'
3'
TM
EpoR probe.
B.
I-6
I-5
I-2
I-1
MS-5
ELM-D
IVP ELM
Spleen
F4-12
Kidney
4.4
2.4
EpoR
1.4
Actin

Figure 8.3.

possible that *in vivo* it may allow stromal cells to respond to increases in the levels of circulating erythropoietin and change the nature of the haemopoietic microenvironment to favour erythropoiesis. However, no experiments have been performed to determine whether a functional epo receptor is expressed on the surface of these cells.

8.3: Summary of phenotypic differences in the ELM system.

Thus, in summary, the ELM system consists of a number of cell lines derived from the same tumour which show marked differences in (a) their stromal cell-dependence, (b) their tumorigenic growth and differentiation properties *in vivo* and (c) their ability to differentiate *in vitro* in response to physiological inducers of erythropoiesis, such as epo. In the case of the I-1 cell line, the differentiation block may be responsible for its increased tumorigenicity and the severe anaemia induced in the host animals. However, I-5, which is also highly tumorigenic, still retains the ability to differentiate, like I-2 and I-6. Interestingly, the I-1 cell line is phenotypically very similar to many of the Friend cell lines generated from mice infected with FV or F-MuLV (see Introduction, section 2.3).

CHAPTER 9: The role of SCF in the stromal cell-dependence of ELM-D#6 cells.

9.1: Expression of *SCF* in MS-5 cells.

A collaborative study was initiated with Dr. Ostertag's group in an attempt to (a) identify erythropoietic growth factors expressed in the MS-5 cells and (b) determine what effect these factors have on the growth of the leukaemic cells by blocking the interaction between the growth factor and its receptor with specific anti-sera. Obvious candidates are IL-3, GM-CSF and SCF, which are unable to support the long-term growth of D#6 cells when presented in a soluble form, either alone or in

combination (Itoh *et al*, 1993), but may represent part of the inductive microenvironment established by the stromal cells. However, using both Northern blot analysis and PCR, *IL-3* mRNA is undetectable in MS-5 cells (Itoh *et al*, 1993): *GM-CSF* mRNA has been detected in this cell line (Suzuki *et al*, 1992), but is absent from other MS-5 cultures that are equally capable at supporting D#6 survival (Itoh *et al*, 1993). The *interleukin-6* and *colony-stimulating factor-1* genes are expressed, but both these factors have no reported direct influence on the survival/growth of erythroid cells, and furthermore, the known receptors for these ligands are not present on D#6 cells (Suzuki *et al*, 1992; Itoh *et al*, 1993).

Examination of MS-5 and ELM cells for *SCF* gene expression was performed using RT-PCR and Northern analysis of RNA samples (Figure 9.1). Using an antisense *SCF*-specific oligonucleotide (*SCF* D1), a reverse transcription reaction was performed, followed by PCR amplification using the *SCF* D1 primer, and a sense oligonucleotide, *SCF* U1. These primers are located either side of a region that is known to be alternatively spliced during *SCF* expression, the inclusion of which allows the proteolytic cleavage of the mature membrane-spanning protein to release soluble SCF (Flanagan *et al*, 1991; Huang *et al*, 1990, 1992). PCR from normal spleen mRNA produced two fragments of the expected size which represent the two *SCF* transcript variants (Figure 9.1B). *SCF* mRNA was readily detectable in the MS-5 feeder cells, but the smaller product was absent. It is likely, therefore, that these cells are able to produce both transmembrane and soluble forms of SCF through the production of a cleavable precursor. Indeed, Ostertag and his colleagues have detected SCF activity in MS-5-conditioned medium that can be blocked with anti-SCF antibodies (Itoh *et al*, 1993). All the other cells tested were *SCF*-negative, although small quantities of PCR product X were generated from ELM-D, D#6 and *in vivo* passaged ELM cells: this was probably a consequence of contaminating stromal cells in these samples.

The PCR product generated from MS-5 RNA was purified and cloned into the pBluescriptII KS+ vector, using the restriction sites incorporated into the PCR

Figure 9.1: Expression of *SCF* in the ELM system.

A: Position of PCR primers (***SCF* U1** and ***SCF* D1**) used to assess *SCF* expression by RT-PCR (see Methods and Materials). Primer sequences are shown in Table 6.2 in the Methods and Materials section. The structure of the cDNA is from Flanagan *et al*, 1991. The position of the proteolytic **cleavage site**, which is alternatively spliced during *SCF* expression, and the putative membrane spanning domain (**TM**), are shown.

B: RT-PCR analysis of *SCF* gene expression in RNA samples from ELM cell lines, MS-5 cells and C3H spleen. RT-PCR reactions were performed as described in the Methods and Materials, the products electrophoresed on a 1% (w/v) agarose gel and visualised using ethidium bromide staining. Molecular weight DNA markers are shown to the right of the gel photograph. **X** represents the 629bp product derived from the transcript containing the proteolytic cleavage site: **Y** is probably from the alternatively-spliced product lacking the exon coding for the cleavage site. The X product generated from MS-5 cell RNA was cloned into pBluescriptII KS+ and partially sequenced.

C: Northern blot of 20μg of RNA samples probed with ^{32}P-radiolabelled *SCF* cDNA fragment X. The position of the 28S and 18S rRNA bands, as determined by ethidium bromide staining, is indicated to the right of the blot. The filter was stripped of the *SCF* probe and hybridised to ^{32}P-radiolabelled *actin* cDNA to assess RNA loading.

Cells tested in the above two experiments were:
I-1, I-2, I-5 and **I-6**:- Stromal cell-independent clones I-1, I-2, I-5 and I-6.
MS-5:- Stromal cell line, MS-5.
D#6:- ELM-D#6 cells growing on live MS-5 cells (6 ELM-D#6 cells per MS-5).
IVP ELM:- *In vivo* passaged ELM tumour cells.
Spleen RNA from a C3H mouse.

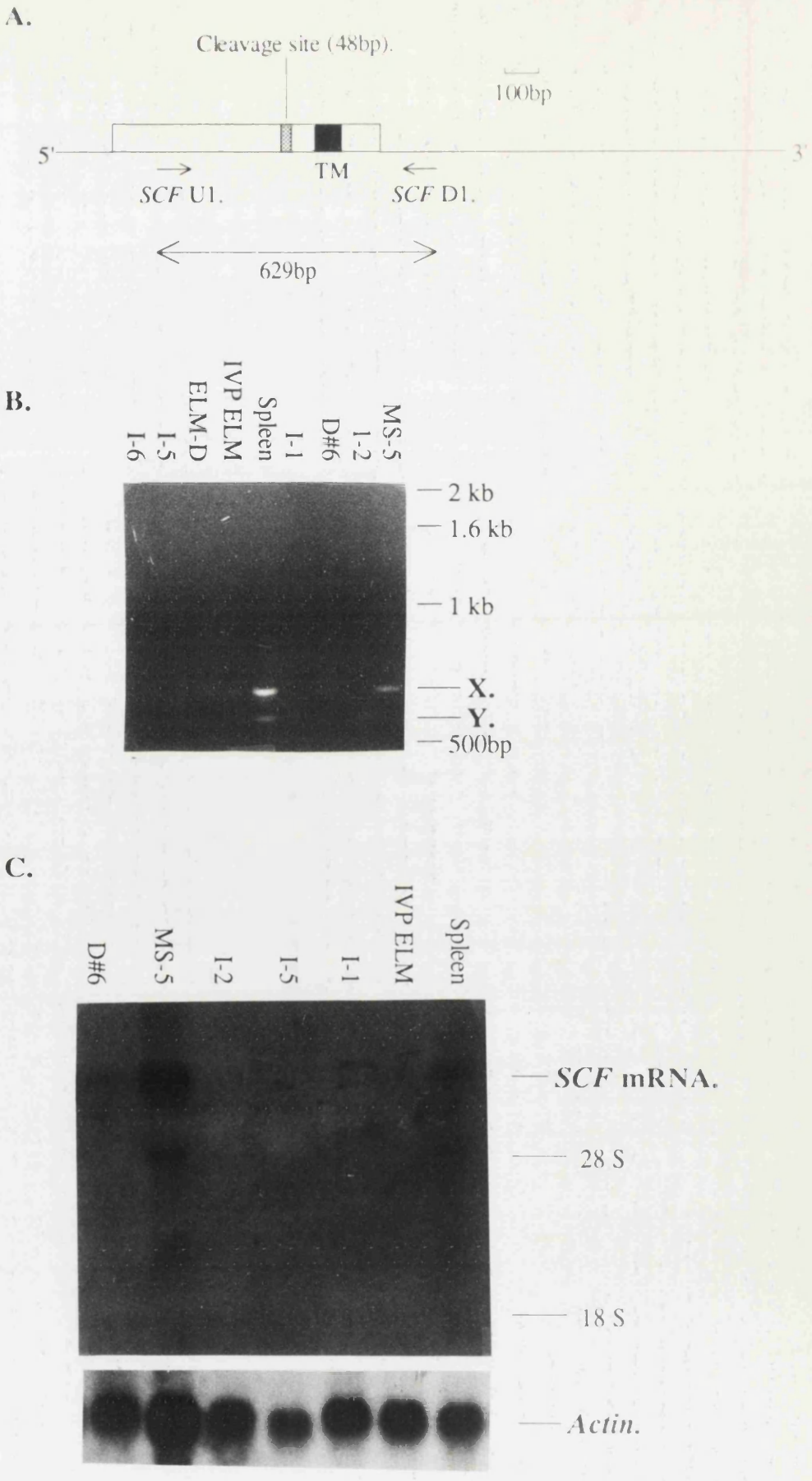

A.
Cleavage site (48bp).
100bp
5'
3'
TM
SCF U1.
SCF D1.
629bp
B.
MS-5
1-2
D#6
I-1
Spleen
IVP ELM
ELM-D
I-5
I-6
2 kb
1.6 kb
1 kb
X.
Y.
500bp
C.
Spleen
IVP ELM
I-1
I-5
I-2
MS-5
D#6
SCF mRNA.
28 S
18 S
Actin.

Figure 9.1.

primers. It was partially sequenced which verified its identity and also confirmed the presence of the region encoding the proteolytic cleavage site (data not shown). The *SCF* cDNA was then radiolabelled and used to probe a Northern blot of various RNA samples. As expected, *SCF* mRNA was only detected in spleen and MS-5 cells, with a faint smear in the *in vivo* passaged ELM tumour cells (Figure 9.1C). The level of RNA loading was demonstrated by reprobing the blot with an *actin* cDNA fragment.

9.2: Expression of *c-kit* in ELM cells.

The RT-PCR procedure was used to generate two cDNA probes for *c-kit*, the gene encoding the SCF receptor, using spleen RNA and the *c-kit*-specific oligonucleotides shown in Figure 9.2A. The RT reaction was primed with *c-kit* D1: the E*kit* probe from the extracellular domain was amplified by PCR using primers *c-kit* U1 and *c-kit* D2, whilst to produce the cytoplasmic domain-encoding I*kit* probe, *c-kit* U2 and *c-kit* D1 were used. These two products were cloned and partially sequenced (data not shown). The E*kit* fragment was initially used to probe the same Northern blot previously hybridised to the *SCF* probe, once it had been stripped of radiolabelled *SCF* probe. ELM-D#6 cells were found to express high levels of the *c-kit* mRNA, as did all the other ELM cells tested, whilst it was absent from MS-5 cells (Figure 9.2B). The low expression of *c-kit* in both normal spleen and erythropoietically-active spleens from anaemic mice, but its relatively high expression in *in vivo* passaged ELM tumour cells, indicates that the ELM erythroleukaemia probably arose from a small population of *c-kit*-expressing haemopoietic cells. Alternatively, the *c-kit* gene may have become deregulated during tumour progression as a consequence of mutation. Uncontrolled expression of growth factor receptors has been associated with other leukaemias, usually as a result of retroviral insertion (Fung *et al*, 1983 (EGF receptor); Lacombe *et al*, 1986 (epo receptor); Gisselbrecht *et al*, 1987 (CSF-1 receptor)). Although Southern blot analysis of ELM cell DNA probed with I*kit* and E*kit* has demonstrated that *c-kit* is not amplified or grossly rearranged

Figure 9.2: Expression of the *c-kit* gene in the ELM system.

A: Position of PCR primers (***c-kit* U1, U2, D1** and **D2**) used to generate two cDNA probes for *c-kit* by RT-PCR (see Methods and Materials). Primer sequences are shown in Table 6.2 in the Methods and Materials section. The structure of the cDNA is from Qiu *et al*, 1988. The portions of the gene encoding the putative membrane spanning domain (**TM**), and the **cytoplasmic** and **extracellular** domains of the protein, are shown. Both the ***Ikit*** and the ***Ekit*** PCR products were cloned into pBluescriptII KS+ and partially sequenced to verify their identity.

B: Northern blot of 20μg of RNA samples probed with ^{32}P-radiolabelled E*kit* cDNA fragment. The position of the 28S and 18S rRNA bands, as determined by ethidium bromide staining, is indicated to the right of the blot. The filter was stripped of the E*kit* probe and hybridised to ^{32}P-radiolabelled *actin* cDNA to assess RNA loading.

Cells tested were:
I-1, I-2, I-5 and **I-6**:- Stromal cell-independent clones I-1, I-2, I-5 and I-6.
MS-5: Stromal cell line, MS-5.
D#6:- ELM-D#6 cells growing on live MS-5 cells (6 ELM-D#6 cells per MS-5).
IVP ELM:- *In vivo* passaged ELM tumour cells.
Spleen:- Normal C3H mouse spleen.
Anaemic spleen:- Erythropoietically-active spleen from a C3H mouse which had been treated with phenylhydrazine to induce anaemia (see Methods and Materials).

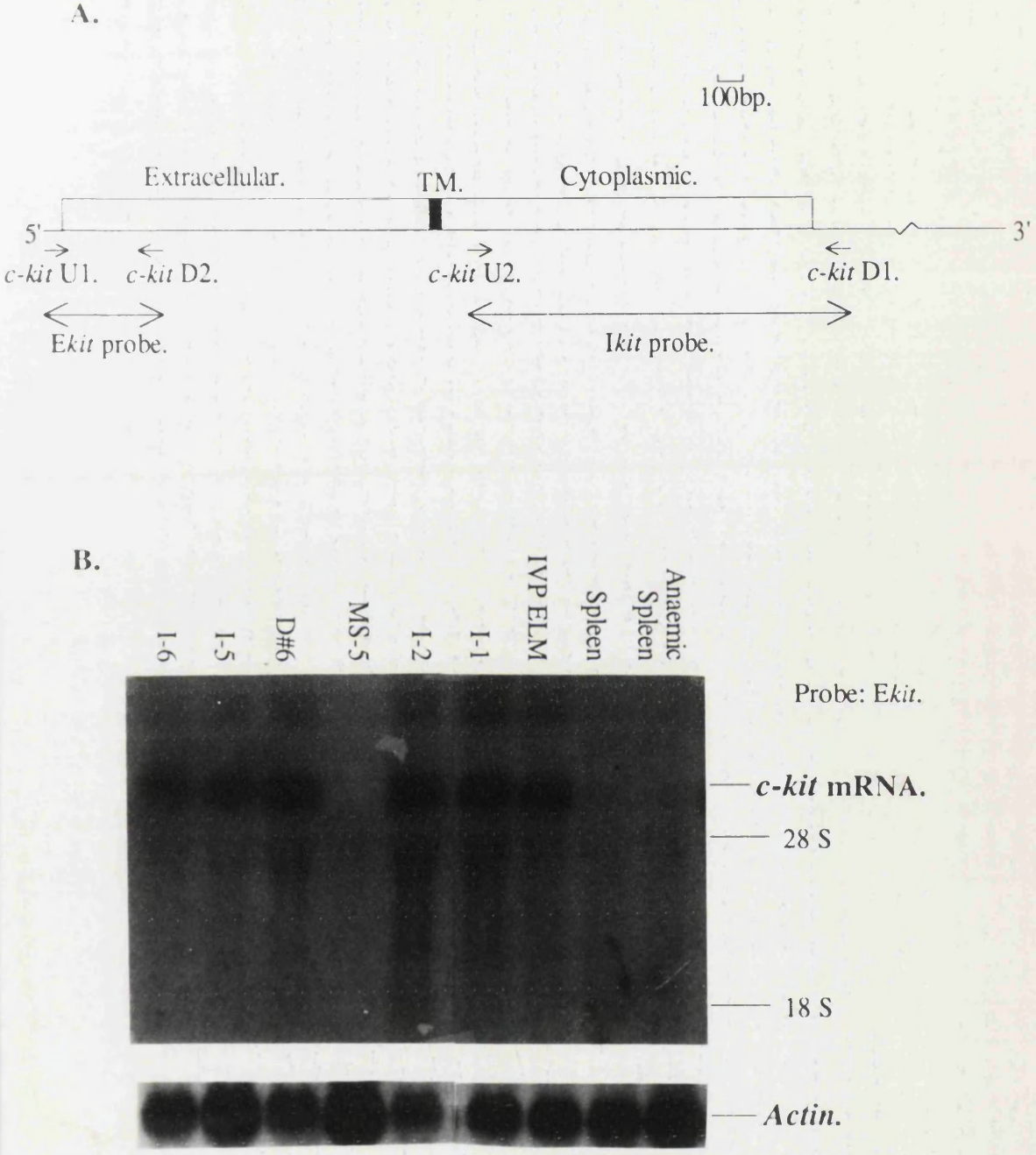

A.
100bp.
Extracellular.
TM.
Cytoplasmic.
5'
3'
c-kit U1.
c-kit D2.
c-kit U2.
c-kit D1.
Ekit probe.
Ikit probe.
B.
I-6
I-5
D#6
MS-5
I-2
I-1
IVP ELM
Spleen
Anaemic Spleen
Probe: Ekit.
c-kit mRNA.
28 S
18 S
Actin.

Figure 9.2.

(data not shown), it still remains a possibility that expression of the gene has become deregulated by an as yet undetected genetic alteration.

9.3: SCF, c-kit and the generation of stromal cell-independent viability.

These results suggested that SCF may be involved in maintaining the long-term viability of D#6 cells. To examine this possibility, Ostertag and his colleagues (Itoh *et al*, 1993) performed two sets of experiments, one using anti-kit and anti-SCF antibodies to block SCF/c-kit interaction, and the second using mutant Sl/Sl^d stromal cells that produce only the soluble form of the ligand (Flanagan *et al*, 1991; Brannan *et al*, 1991; Huang *et al*, 1992). They have demonstrated that whilst the antibodies do not prevent adhesion of D#6 cells to MS-5 cells, they do significantly reduce the growth of D#6 cells in a D#6/MS-5 coculture by greater than 75% relative to untreated controls. The Sl/Sl^d stromal cells, in a similar fashion to SCF-supplemented medium, are able to support transient, but not long-term growth of D#6 cells. It was concluded from these results that the TM SCF/c-kit interaction plays a crucial role in blocking cell death and promoting erythroid progenitor cell survival, an interaction which can not be adequately replaced by binding of the soluble form of SCF to c-kit.

This raised the possibility that stromal cell-independent growth could arise as a consequence of a mutation that constitutively activates the signalling pathway stimulated by TM SCF/c-kit association. This could occur by the autocrine production of SCF, and indeed, growth factor production by leukaemic cells has been previously reported (Miyanomae *et al*, 1985; Sawada *et al*, 1986; McDonald *et al*, 1987; Meeker *et al*, 1990). However, as shown in Figure 9.1B and C, cell lines I-1, I-2, I-5 and I-6 produce no *SCF* mRNA that is detectable by Northern blot and RT-PCR analysis. An alternative hypothesis involves the constitutive ligand-independent activation of the receptor by mutation. The only known oncogenic form of *c-kit*, *v-kit*, contains a large deletion which removes the portion known to encode the extracellular and membrane-spanning domains of the protein (Besmer *et al*, 1986). However, in the four stromal cell-independent cell lines tested (I-1, I-2, I-5 and I-6)

the I*kit* and E*kit* probes (Figure 9.2A) both hybridise to the full-length transcript also seen in D#6 cell RNA, effectively excluding similar deletions in the generation of stromal cell-independent mutants (Figure 9.2B and data not shown). Furthermore, no gross rearrangements are detected with either probe on Southern blots of *ECo* RI or *Bam* HI-cleaved DNA from the I-1 and I-2 cell lines (data not shown). However, these preliminary experiments do not exclude the possibility that subtle point mutations, akin to those reported in the CSF-1 receptor in patients with acute myelogeneous leukaemia (Ridge *et al*, 1990), may cause ligand-independent signalling and promote stromal cell-independent survival.

CHAPTER 10: *p53* status.

10.1: Detection of a *p53* mutation in the ELM-D cells.

Studies of *p53* genes in Friend cell lines have demonstrated that mutation, accompanied by loss of the remaining normal allele, appears to be an obligatory step in the progression of Friend erythroleukaemia, irrespective of whether it is induced with FV-A, FV-P or F-MuLV (reviewed in Ben-David and Bernstein, 1991). Friend cell lines usually exhibit three phenotypic characteristics: (a) high tumorigenicity *in vivo*, (b) an inability to differentiate in response to epo, and (c) stromal cell- and growth factor-independent growth *in vitro*. By examining the *p53* status within the ELM system, which shows variation with respect to all these properties, it should prove possible to determine whether mutation of the gene correlates with the acquisition of a particular phenotype.

As an initial screen for mutation, Southern blots were performed to compare the structure of the *p53* genes in the highly tumorigenic, differentiation-resistant I-1 cells in comparison normal spleen tissue. Similar experiments had successfully identified *p53* mutations in Friend cell lines (Mowat *et al*, 1985; Rovinski *et al*, 1987; Hicks and Mowat, 1988; Munroe *et al*, 1988). However, using five different

restriction enzymes and a full-length murine *p53* cDNA probe, no differences were observed, demonstrating that no gross rearrangements or insertions had occurred at the *p53* locus (data not shown). Furthermore, Northern blot analysis of RNA from a number of the ELM cell lines showed that a full-length transcript was being expressed at levels comparable to normal spleen tissue (data not shown).

To test for expression of the protein, ELM-D cells growing on a MS-5 cell layer, were stained with the anti-p53 antibody PAb 246 which recognises an epitope spanning amino acid residues 88 to 109 (Yewdell *et al*, 1986; Figure 10.2). The protocol employed a biotin-conjugated horseradish peroxidase moiety which deposits a brown stain at the position of p53:PAb 246 complexes when incubated with its substrate, diamino benzidine (see Methods). As shown in Figure 10.1A, the nuclei of the ELM-D cells stain well, whilst the fibroblast-like MS-5 cells underneath are relatively unstained. In comparison, there was essentially only uniform background staining of cells in a control coculture which had been treated identically, except without the inclusion of PAb 246 (Figure 10.1B). The intensity of the ELM-D staining with this antibody is consistent with the production of a large quantity of p53 by these cells, and although PAb246 is not specific for mutant p53 proteins, stabilisation and accumulation of p53 protein is an indication that it is in the mutant conformation (Oren *et al*, 1981; Finlay *et al*, 1988). This result justified a search for a mutation at the nucleic acid level.

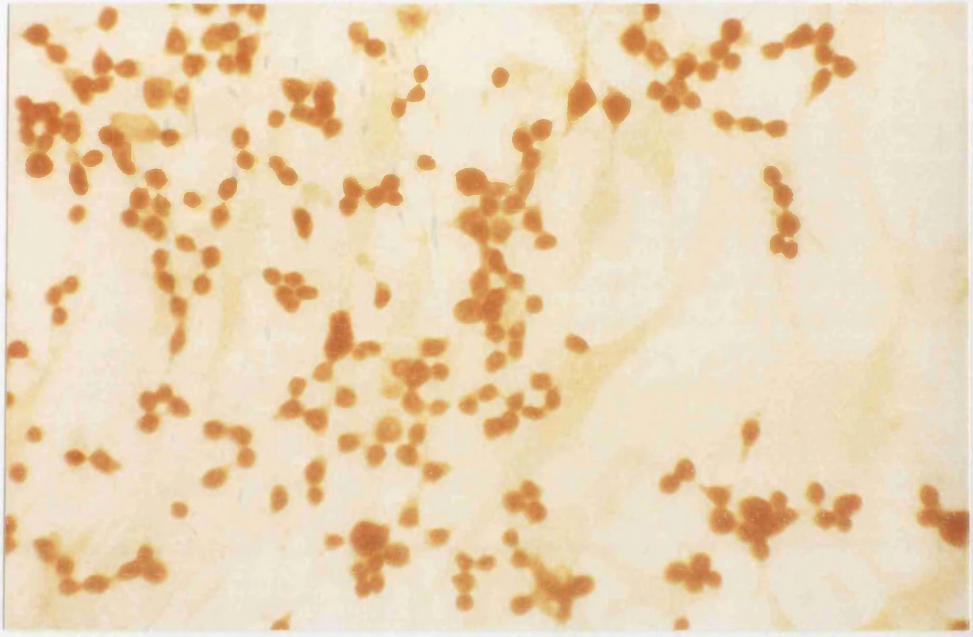
In an attempt to identify a mutation in the coding region of the *p53* gene, small portions of cDNA were amplified by RT-PCR from ELM-D RNA, and then sequenced. In each reaction, RNA from the spleen of a normal C3H mouse was used as a wild-type control. Initially, a reverse transcription reaction was performed, primed by an antisense oligonucleotide (*p53* D1) that hybridises to a sequence in the 3' end of the *p53* transcript (Figure 10.2). Small aliquots from this reaction were then subjected to PCR using the *p53* D1 primer, and a sense primer *p53* U1 (Figure 10.2), that hybridises near the 5' end of the *p53* coding region. This generated a fragment, ~1.15kb in length, containing nearly all the coding sequence of the gene, including

Figure 10.1: ELM-D cells stained with the anti-p53 antibody, PAb246.

A: ELM-D cells growing on MS-5 cells at approximately 75% confluence were fixed and treated with the anti-p53 antibody PAb246. The position of the p53:PAb246 complexes was determined using horse anti-mouse antibodies linked to a horseradish peroxidase moiety: subsequent treatment with diamino benzidine deposits a brown stain as a result of enzymatic cleavage by the peroxidase (see Methods and Materials). The intense staining over the nuclei of the ELM-D cells is apparent, whilst the MS-5 stromal cells growing underneath stain only very weakly.

B: Control cells treated identically, except that the incubation step with the PAb246 antibody was omitted.

A.



B.

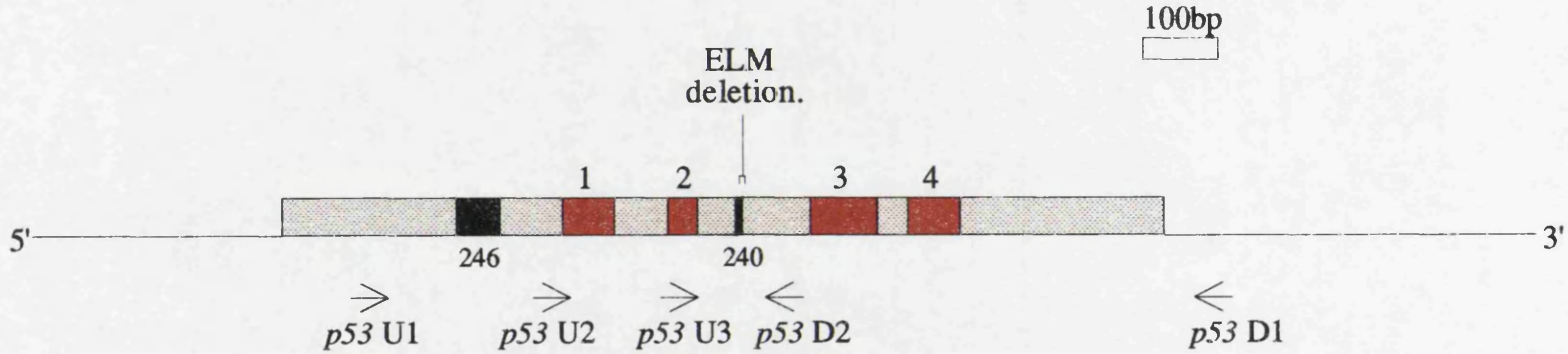
Figure 10.1.

Figure 10.2: Position of *p53* primers, 'hot-spots' and epitopes.

Primers: The position (indicated by the arrows) and the sequence of the five primers (***p53* U1, U2, U3, D1** and **D2**) used to generate and sequence a PCR product spanning the ELM mutation.
'Hot-spots': The position of the four regions of the *p53* gene most commonly targeted for mutation in human tumours is represented by the four **red boxes** (from Hollstein *et al*, 1991).
Epitopes: The position of the epitopes for the two anti-p53 antibodies, **PAb 240** and **PAb246**, are shown as **black boxes** (from Stephen and Lane, 1992, and Yewdell *et al*, 1986)

The position of the 18bp deletion, identified in ELM cells in this thesis, is also shown.

The structure of the *p53* cDNA and the sequence of the primers is from Bienz *et al*, 1984.



Primer sequences (U: sense; D: antisense).

p53 U1 : 5' ACCTCACTGCATGGACGATCTGTTGCT 3'
p53 U2 : 5' TTCCACCTGGGCTTCCTGCAGTCT 3'
p53 U3 : 5' CTCCTCCCCAGCATCTTATCC 3'
p53 D1 : 5' CAGCAGAAGGGACCGGGAGGATTG 3'
p53 D2 : 5' CTATTACACATGTACTTGTAGTGGATG 3'

Coding region.

Mutation 'hot-spots'.

240 Epitope for anti-p53 antibody PAb240.

246 Epitope for anti-p53 antibody PAb246.

Figure 10.2.

the four mutational 'hot-spots'. *p53* D1 is unable to hybridise to the *p53* pseudogene: this prevents amplification of this sequence from possible genomic DNA contamination in the RNA samples.

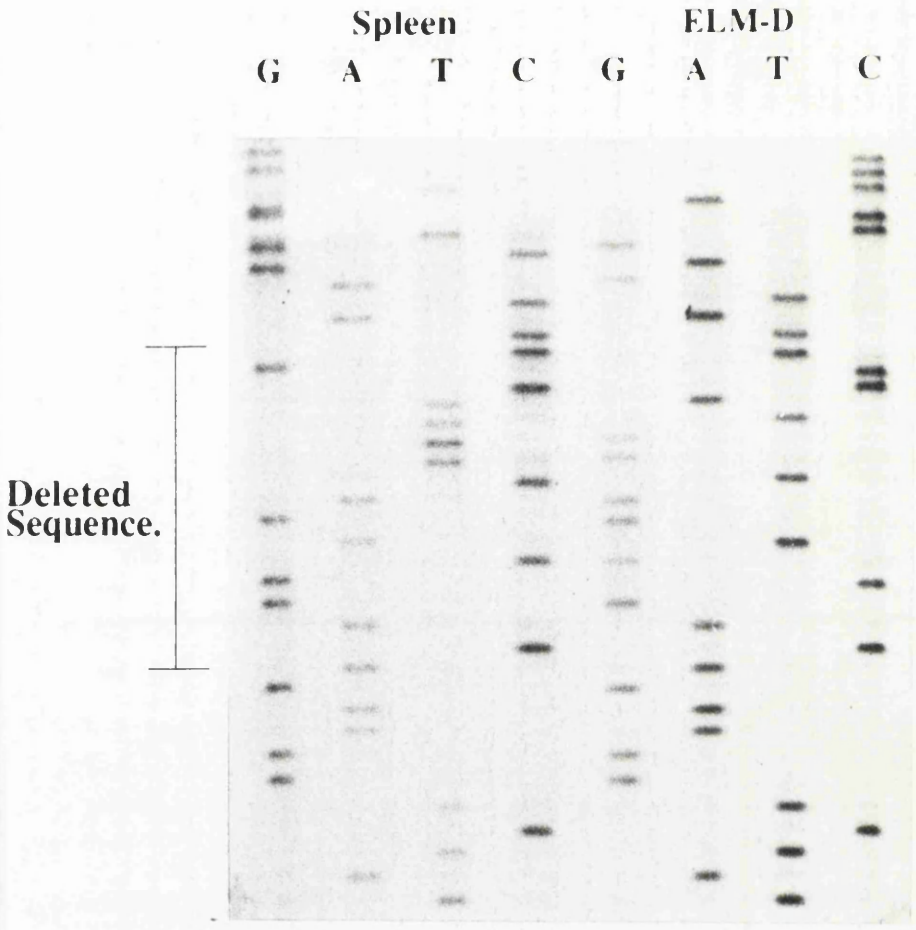
Aliquots from the *p53* U1/*p53* D1-primed reaction were then used as a template for a second round of amplification which employed 'nested' primers that hybridise to sequences between *p53* U1 and *p53* D1. This generated relatively large quantities of specific regions of the coding region, which were then sequenced using a third primer that hybridised between the 'nested' primers. Initially, the mutation 'hot-spots' were targeted, but neither 'hot-spot' 1 nor 3 (depicted in Figure 10.2) contained a mutation in ELM-D cells. However, when the products of a further PCR reaction, primed by *p53* U2 and *p53* D2 (Figure 10.2), were run on a 3% agarose gel, the fragment from ELM-D RNA appeared to be smaller than the fragment from spleen RNA suggesting that it contained a small deletion (data not shown). This was confirmed by direct sequencing using the *p53* U3 primer (Figure 10.2), which demonstrated that 18bp had been lost from the ELM-D cDNA (Figure 10.3) between 'hot-spots' 2 and 3 and encoded by exon 6. The deletion maintained the reading frame and did not introduce a stop codon: therefore, a nearly full-length protein lacking amino acid residues 206 to 211 should be produced by the transcript. The absence of any wt*p53* sequence in the ELM-D PCR product suggested that no wt*p53* mRNA was present in ELM-D cells. Interestingly, adjacent to each breakpoint of the deletion is the sequence ACAG; short repeats of this kind are a common feature of spontaneous deletion mutations, and are probably important in the mechanism of deletion (Nalbantoglu et al, 1986). In fact, all short deletions of >2bp identified in the human *p53* gene to date, contain direct repeats of between 2 and 8bp in the close vicinity of the deletion (Jego *et al*, 1993).

10.2: All cells of the ELM system are hemizygous for the mutation.

Fortunately, a *Dra* III restriction site (recognition sequence: CACNNNGTG) is lost as a consequence of the deletion and this was exploited diagnostically to (a)

Figure 10.3: Sequence analysis of the *p53* mutation present in ELM-D cells.

RT-PCR with primers *p53* U2 and *p53* D2 was used to generate a 394bp product spanning the central portion of the *p53* cDNA from ELM-D and normal C3H spleen cells (see Methods and Materials for RT-PCR protocol). Each fragment was sequenced directly using the *p53* U3 primer shown in figure 10.2, which hybridised to a region between the two primers used in the PCR reaction. The 18 base pairs present in the wild-type spleen PCR product, but deleted in the ELM-D product, are shown by the brackets to the left of the sequence gel autoradiograph and again in the sequence written below. The **ACAG** repeats are boxed, and the recognition site for the ***Dra* III** restriction endonuclease (CACNNNGTG), present in the spleen product only, is marked. p53 amino acid sequence and numbering are from Bienz *et al*, 1984.



Dra III site.

204 213

glu asp arg gln thr phe arg his ser val

GAA GAC AGG CAG ACT TTT CGC CAC AGC GTG spleen

GAA GAC AGC GTG ELM-D

glu asp ser val

Figure 10.3.

check the other ELM cells for the mutation, and (b) assess whether a normal allele was present. A Southern blot of *Dra* III-digested DNA from cells of the ELM system was prepared and hybridised to a radiolabelled probe from intron 7 of the *p53* gene (Figures 10.4A and 10.5). This probe was generated by PCR from normal spleen DNA (see Materials and Methods) and has the advantage of not hybridising to the *p53* pseudogene, making interpretation of the results simpler. The probe hybridised to a single 5.8kb band in normal spleen DNA; a larger 11kb band predominated in DNA from the ELM cells, whilst the 5.8kb band was absent (Figure 10.4A). Since an intron 2 probe from the 5' side of the *Dra* III site (Figure 10.5) hybridised almost exclusively to a 5kb band in *Dra* III-cleaved spleen DNA (Figure 10.4B, extreme right-hand lane), it was assumed that the 11kb species represented alleles in which the *Dra* III site was absent due to the 18bp deletion. The presence of this band in DNA samples from the *in vivo* passaged tumour cells and all *in vitro* cell lines demonstrates that the 18bp deletion in the *p53* gene was an early event involved in generating the original erythroleukaemia, and is not associated with phenotypic changes of the cell lines *in vitro*. Likewise, the absence of the wild-type 5.8kb band shows that this mutation was accompanied by loss of the remaining normal allele before cells were established in culture.

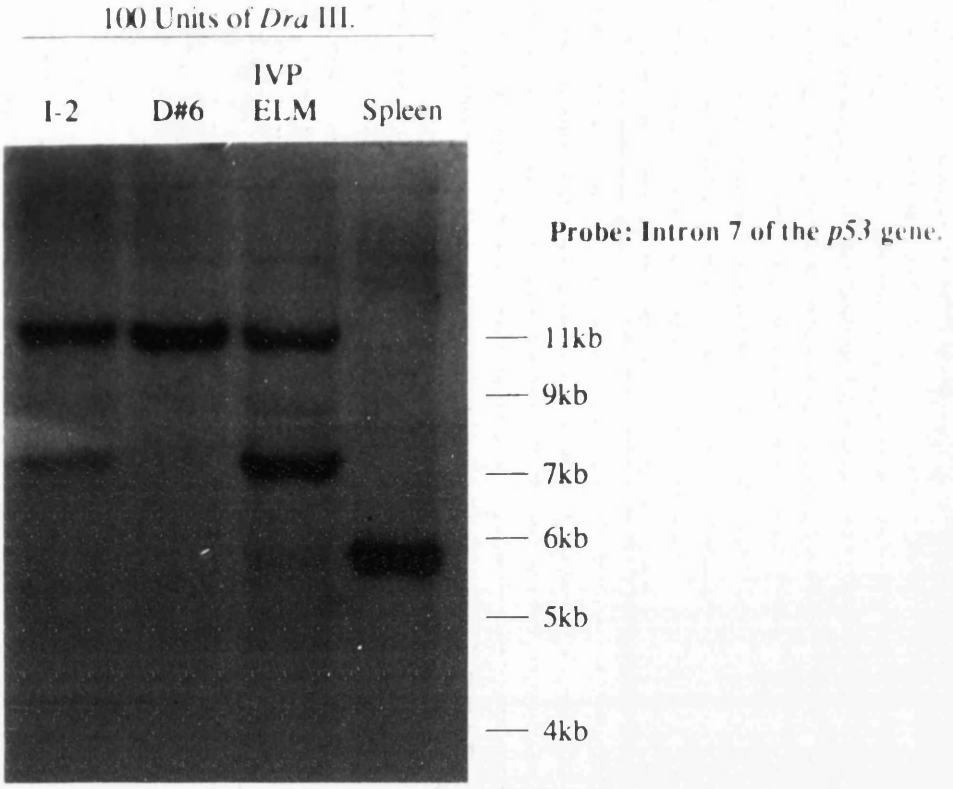
However, an additional band of 7kb was also apparent in the DNA from I-1 and *in vivo* passaged tumour cells (Figure 10.4A). It seemed unlikely that this represented a second mutational event in the *p53* gene that creates a *Dra* III site in these cells, but a more plausible explanation was that it was an artefact generated during digestion of the DNA. One possibility was that there was a sequence, approximately 1.2kb 5' to the *Dra* III site of exon 6, that could be cut inefficiently by *Dra* III (Figure 10.5). This was confirmed by digesting several 20μg aliquots of normal spleen DNA with increasing concentrations of *Dra* III and preparing a Southern blot. Hybridising to a radiolabelled *p53* intron 2 fragment, showed that as the concentration of *Dra* III was increased, the predominant band shifted from 5kb to 3.8kb (Figure 10.4B). In fact, there are two sequences in exon 4 (CACNNNATG and

Figure 10.4: Southern blot analysis of the *p53* gene in *Dra* III-cleaved genomic DNA samples.

A: 20μg of genomic DNA from C3H spleen, *in vivo* passaged ELM tumour cells (IVP ELM), ELM-D#6 cells growing on MS-5 stromal cells (D#6) and ELM-I-2, was cleaved with 100 Units of *Dra* III and a Southern blot prepared according to the Methods and Materials. This was hybridised to intron 7 of the *p53* gene previously isolated by PCR from spleen genomic DNA using primers and PCR conditions described in the Methods and Materials, section 6.12. Exposure time: 3 days. The position of molecular weight DNA markers electrophoresed adjacent to the DNA samples are shown to the right of the autoradiograph.

B: 20μg samples of genomic DNA from C3H spleen digested with various concentrations of *Dra* III (given at the top of the autoradiograph) were used to prepare a Southern blot (see Methods and Materials). This was hybridised to intron 2 of the *p53* gene previously isolated by PCR from spleen genomic DNA using primers and PCR conditions described in the Methods and Materials, section 6.12. Exposure time: 5 days. The position of molecular weight DNA markers electrophoresed adjacent to the DNA samples are shown to the right of the autoradiograph.

A.



B.

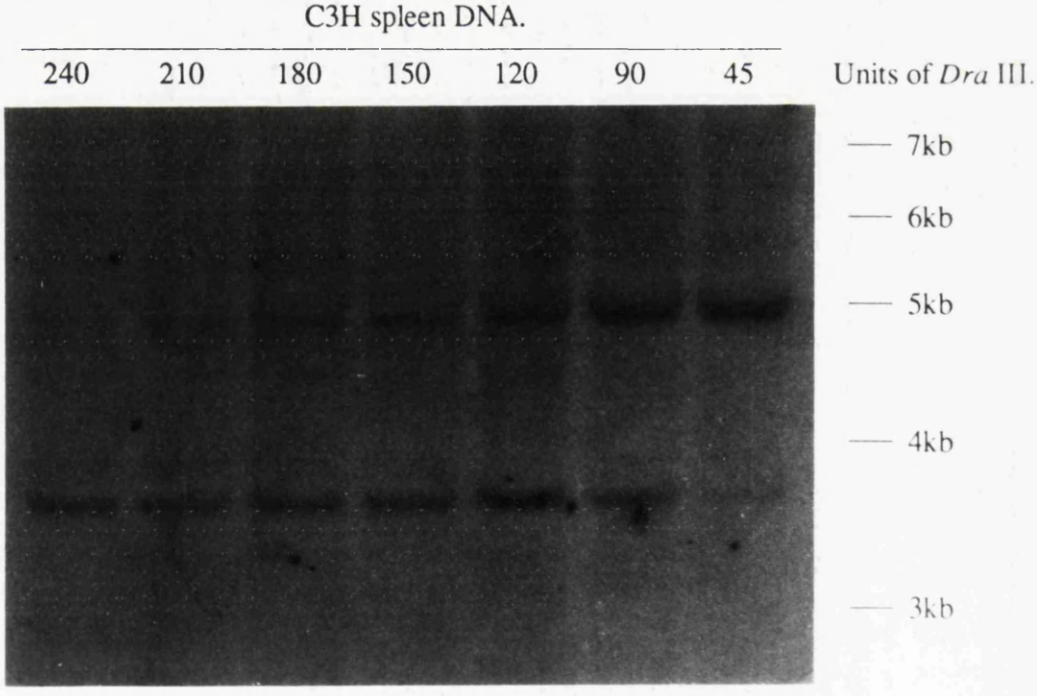


Figure 10.4.

Figure 10.5: Genomic organisation of the *p53* gene showing the position of primers, probes and *Dra* III cleavage sites important in this study.

Primers: The position and sequence of the ***p53* U4** and ***p53* D4** primers are shown. These were used in the PCR reactions involved in the generation of Figure 10.6.
Probes: The **intron 2, intron 7** and ***Sst* II** cDNA probes are shown in red. These were used on various Southern blots performed in this study (see text for further details).
***Dra* III cleavage sites**: The site of known *Dra* III cleavage sequences is shown above the gene structure, along with the size of the fragments formed upon digestion. The putative weak *Dra* III site in exon 4, identified in this study (see text), is also shown.

The position of the exons and introns (coding; filled boxes: non-coding; empty boxes) is derived from Bienz *et al*, 1984. This reference was also used to provide the sequence for the two primers.

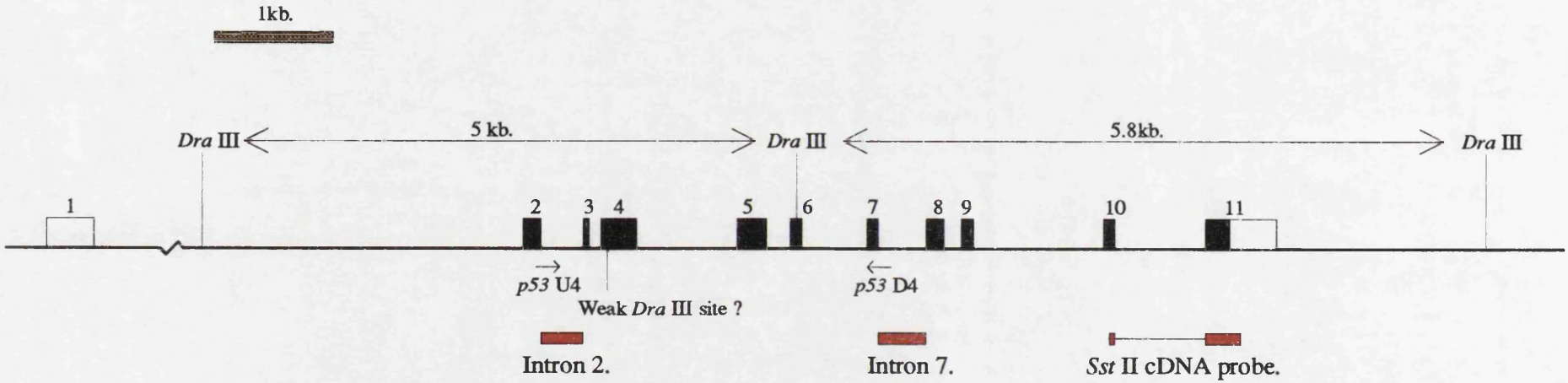

1kb.
Dra III
5 kb.
Dra III
5.8kb.
Dra III
1
2
3
4
5
6
7
8
9
10
11
p53 U4
p53 D4
Weak Dra III site ?
Intron 2.
Intron 7.
Sst II cDNA probe.
Coding exons.
Non-coding exons.
Probes.
Primer sequences.
p53 U4 : 5' TCAGGCTTATGGAAACTGTGAGTGGAT 3'
p53 D4 : 5' CTTCCAGTGTGATGATGGTAAGGATAG 3'

Figure 10.5.

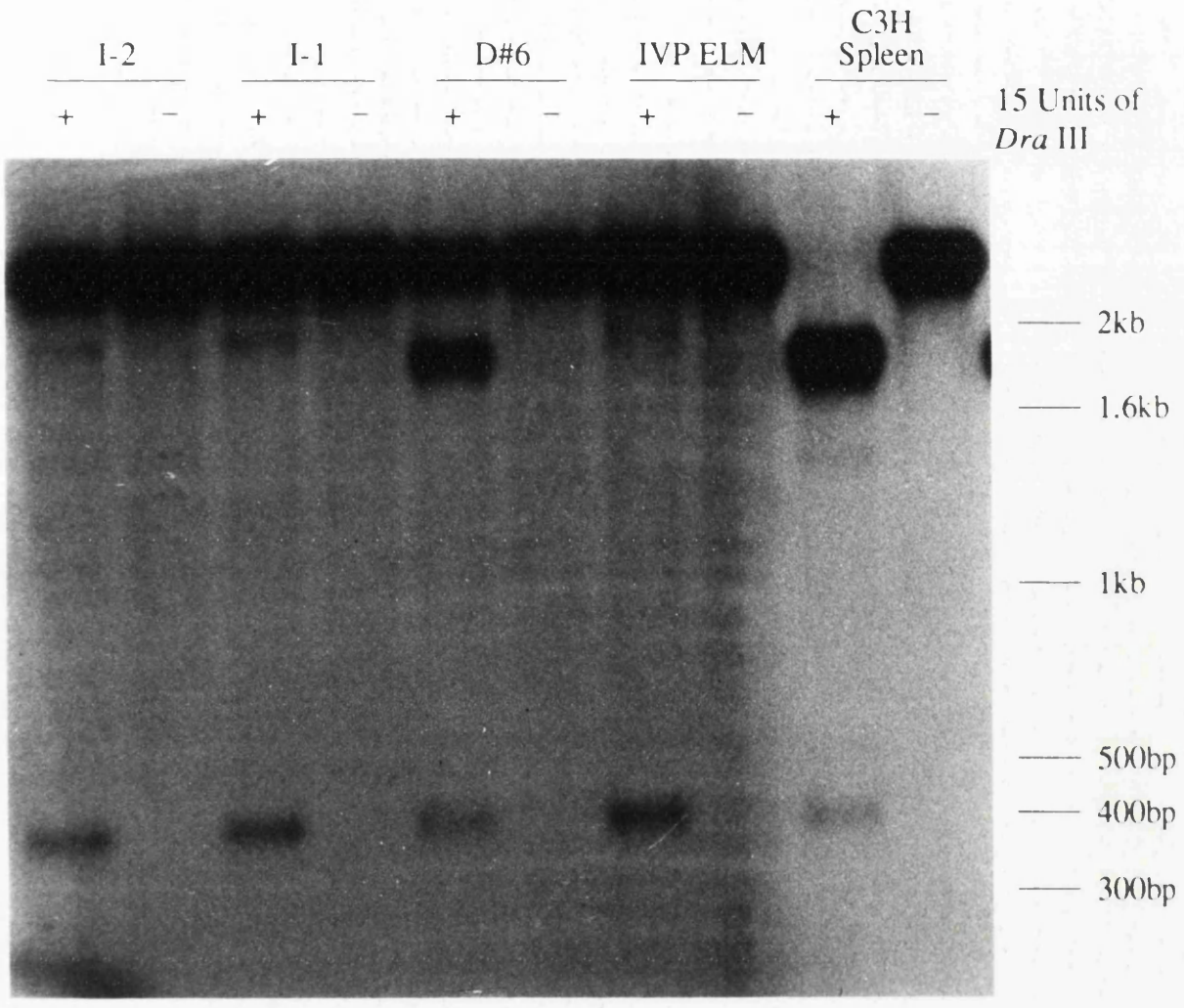
CTCNNNGTG), which are approximately 1.2kb 5' to the *Dra* III site in exon 6, which have 8/9 homology to the *Dra* III recognition sequence (CACNNNGTG), and may possibly act as weak *Dra* III cleavage sites. The variation in the intensity of the 7kb band in the ELM samples in Figure 10.4A, was probably due to different salt concentrations in the DNA preparations which was known to affect the efficiency of cleavage at *Dra* III sites (data not shown). Alternatively, DNA methylation variations between samples may affect the ability to cleave these putative *Dra* III sites.

PCR has been used to confirm the conclusions drawn from the Southern blot analysis. Primers *p53* U4 and *p53* D4, which anneal to sequences either side of the deletion and not to the pseudogene (Figure 10.5), were used to generate a 2.3kb fragment from DNA from cells in the ELM system. 100ng was then incubated with or without *Dra* III, electrophoresed on a 1% agarose gel and blotted onto a nylon membrane. Hybridisation with an intron 2 probe, radiolabelled with ^{32}P, shows that none of the PCR products from any of the ELM cells were cleaved by *Dra* III, whilst the same fragment from normal spleen DNA digested completely to the expected 1.8kb product (Figure 10.6). (The small amount of D#6 product that was cleaved (~10-15%) was most probably due to contaminating feeder cell DNA in the D#6 genomic DNA preparation). In a control experiment using an equimolar mixture of spleen and ELM PCR products, 50% digested as expected, a result which demonstrates that lack of cleavage by *Dra* III was due to loss of the recognition site, and not because of inhibition of the enzyme by contaminants in certain PCR product preparations (data not shown). A smaller band of low intensity was seen comigrating with the 400bp marker in all the cleaved products. Again, this was probably a consequence of digestion at the weak *Dra* III site identified by Southern blotting.

Loss of the normal allele from a developing tumour cell can occur by a number of distinct mechanisms (Hansen and Cavanee, 1987) that produce one of two results: (a) homozygosity i.e. two copies of the same mutant allele, or (b) hemizygosity, when only one copy of the mutant allele remains. To distinguish between these two possibilities the Southern blot originally probed with intron 7

Figure 10.6: Southern blot analysis, after incubation with or without *Dra* III, of PCR products spanning the ELM deletion in the *p53* gene.

PCR reactions, primed with the p53 U4 and p53 D4 oligonucleotides (Figure 10.5), were performed, as described in Methods and Materials, using genomic DNA from the following cells:
I-1 and **I-2**:- Stromal cell-independent clones I-1 and I-2.
D#6:- ELM-D#6 cells growing on live MS-5 cells (6 ELM-D#6 cells per MS-5).
IVP ELM:- *In vivo* passaged ELM tumour cells.
C3H spleen:- Normal C3H mouse spleen.
100ng of the PCR product from each sample was incubated with or without 15 Units *Dra* III for 2 hours, electrophoresed on a 1% (w/v) agarose gel and a Southern blot prepared. This was hybridised to a *p53* intron 2 probe. Exposure time: 12 hours. The position of molecular weight DNA markers electrophoresed adjacent to the DNA samples are shown to the right of the autoradiograph.



Probe: Intron 2 of the *p53* gene.

Figure 10.6.

(Figure 10.4A) was stripped and reprobed with a *Sst* II fragment from the 3' region of the *p53* cDNA (Figure 10.5 shows the position of the probe; Figure 10.7A shows the autoradiograph of the blot). This generated a result identical to Figure 10.4A, except that the pseudogene band is also apparent (Figure 10.7A). Scanning densitometry of this autoradiograph, using the pseudogene band as an internal loading control, and combining the intensity of the 11kb and 7kb bands in the ELM lanes, demonstrated that the ELM cells are hemizygous: only one mutant allele is present (Figure 10.7B). The higher gene/pseudogene ratio observed in the IVP ELM and D#6 samples is probably a consequence of a small number of contaminating normal spleen cells and MS-5 cells, respectively.

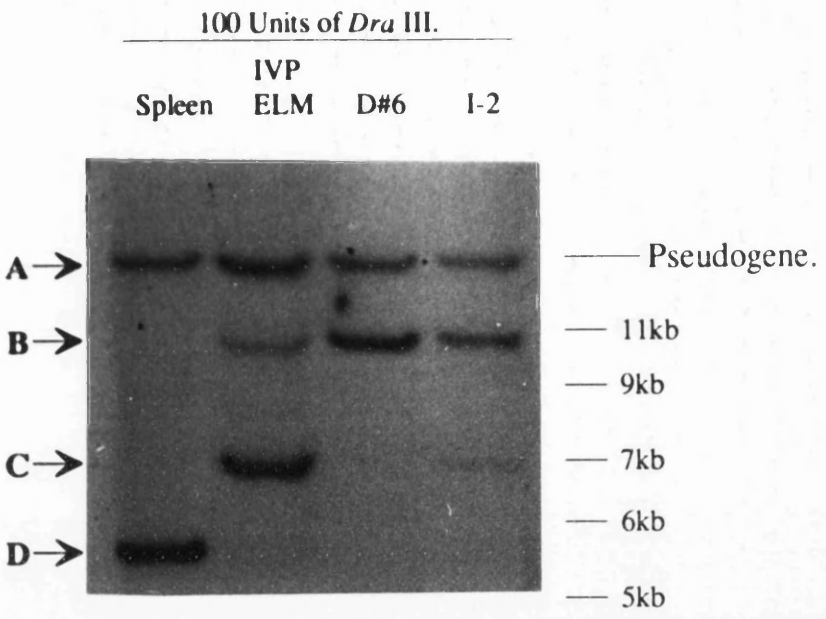
Thus, in conclusion, all cells of the ELM system are hemizygous for a *p53* allele containing an 18bp deletion. Mutation of the *p53* gene is not directly responsible for any of the phenotypic differences between *in vitro* ELM cell lines, but was probably an important step in the evolution of the original erythroleukaemia. The deletion described here does not disrupt the reading frame of the transcript, or introduce a stop codon, thus a nearly full-length protein will be translated that lacks amino acid residues 206 to 211, inclusive. In-frame deletions are a relatively unusual mode of *p53* inactivation: any deletions that do occur usually shift the reading frame of the transcript and translate to a truncated protein lacking the important carboxy-terminal oligomerisation domain (Hollstein *et al*, 1991; Caron de Fromentel and Soussi, 1992; Jego *et al*, 1993). Also, the deletion is outside the previously defined mutational 'hot-spots' of the *p53* gene, although point mutations and frameshifts have been identified in the region of the gene deleted in ELM cells. Interestingly, the ELM deletion removes part of the epitope for the PAb 240 antibody which becomes exposed, presumably as a result of a conformational change, in a large number of different p53 mutant proteins (Figure 10.2; Stephen and Lane, 1992). One would assume, therefore, that the structural integrity of this region is necessary for wtp53 function.

Figure 10.7: *p53* copy number analysis in the ELM system.

A: The Southern blot previously probed with intron 7 of the *p53* gene (Figure 10.4A) was stripped of the radioactivity and hybridised to a ^{32}P-radiolabelled *Sst* II fragment from the 3' end of the *p53* cDNA (see Figures 10.2 and 10.5 for the position of the fragment). Exposure time: 3 days. The invariant **pseudogene** band is indicated. All bands have been given a number, shown with an arrow to the left of the autoradiograph, to permit easy cross-reference with the scanning densitometry table shown in B. The position of molecular weight DNA markers electrophoresed adjacent to the DNA samples are shown to the right of the autoradiograph.

B: The autoradiograph shown in panel A was subjected to scanning densitometry, and a value calculated for each band representing the optical density multiplied by the area of the band under study. These figures were then used to determine the ratio of gene copies to pseudogene copies. This ratio, by comparison to the spleen sample which was assumed to have two *p53* genes per cell, gave an estimate as to the number of copies present in the various ELM cells. The position of the gene bands observed in the *Dra* III Southern in panel A determined whether they were mutant or normal alleles.

A.



Probe: 3' end of *p53* cDNA (*Sst* II fragment).

B.

	Optical density (OD) x Band area (mm^2).			
Band.	**Spleen.**	**IVP ELM.**	**D#6.**	**I-2.**
A. (pseudogene).	6.30	8.49	6.47	4.39
B. (mutant).	0	3.65	10.83	6.04
C. (mutant).	0	11.46	0	0.56
D. (wild-type).	18.67	0	0	0

Gene / Pseudogene	**2.96**	**1.78**	**1.67**	**1.50**
	↓	↓	↓	↓
	2 *wtp53* alleles.	**1 mutant *p53* allele.**	**1 mutant *p53* allele.**	**1 mutant *p53* allele.**

Figure 10.7.

CHAPTER 11: The role of *ets*-related genes in the ELM system.

11.1: Screening I-1 and I-2 for ets proteins.

Studies on Friend erythroleukaemia have highlighted the importance of *ets*-related genes in the disruption of normal erythropoiesis. Induction with SFFV invariably causes the deregulated expression of the *Spi-1* gene (Moreau-Gachelin *et al*, 1988, 1989, 1990), whilst erythroleukaemias developing in newborn mice infected with F-MuLV are associated with over-expression of the related *Fli-1* gene (Ben-David *et al*, 1991); in both cases this is due to the selection during tumour progression of a particular retroviral integration event at a position upstream of their promoter sequences. However, the exact role of these genes in the manifestation of the leukaemic phenotype is not known. The ELM system, which displays a number of phenotypic variants with respect to differentiation and growth properties, may permit a correlation to be drawn between the acquisition of a particular phenotype and expression of *ets*-related genes.

Since the ELM erythroleukaemia was reportedly generated by irradiation (Itoh *et al*, 1988a), the close correlation between infection with a specific retrovirus and deregulated expression of *Spi-1* or *Fli-1* may not be observed. In fact, expression of other *ets*-related genes may be activated in their place to serve a similar function. To screen for this, three ets-specific oligonucleotides, PEA3, PU-1 and E74 (see table 6.4 in the Methods section), were used in electrophoretic mobility shift assays (EMSAs). These oligonucleotides, which bind to a wide spectrum of ets proteins, were used to give a profile of ets DNA binding activity present within the ELM system. PEA3 is derived from the oncogene responsive domain of the polyoma virus enhancer (Wasylyk *et al*, 1989), and in EMSAs is known to bind to *in vitro*-translated murine c-ets-1, c-ets-2 and elk-1 proteins, and also to the product of the recently cloned *PEA3* gene (Wasylyk *et al*, 1990; Rao and Reddy, 1992; Xin *et al*, 1992). The PU-1 sequence, derived from the SV40 enhancer (Klemsz et al, 1990), binds to the product of the *PU-1* gene which proved to be identical to *Spi-1* (Goebl *et al*, 1990). The

product of the closely-related *Spi-B* gene is also able to bind to the PU-1 sequence *in vitro* (Ray *et al*, 1992). The E74 oligonucleotide contains the consensus sequence required for binding to the Drosophila E74 protein (Urness and Thummel, 1990); it also binds to the human erg protein, but not c-ets-1 or c-ets-2 (Reddy and Rao, 1991). In all of these cases, if an *ets* gene product shows an ability to bind to a particular sequence in EMSAs, then exogenous over-expression of that gene *in vivo* is able to activate transcription from a promoter containing tandem repeats of the sequence (Klemsz *et al*, 1990; Reddy and Rao, 1991; Ray *et al*, 1992; Xin *et al*, 1992).

Nuclear protein extracts were prepared from the I-1 and I-2 cell lines (see Materials and Methods), chosen because of their different routes of isolation and phenotypic variation: a similar preparation was generated from *Spi-1*-expressing F4-12 cells to be used as a control. To determine the quality of these extracts, and to give a rough estimate of protein concentration, they were tested in an EMSA for the presence of proteins able to bind to the αP3A oligonucleotide, containing the CCAAT box of the mouse α-globin promoter (see Materials and Methods; Plumb *et al*, 1989). This sequence is able to bind to a protein which is thought to be present in a large number, if not all, cell types (Cohen *et al*, 1986). The three extracts generated a retarded complex in this assay, although I-1 was approximately threefold more intense than F4-12 and I-2 (data not shown): hence, in subsequent EMSAs, for every 1μl of I-1 protein used, 3μl of the I-2 and F4-12 extract was used. In the following experiments, retarded complexes that were 'specific' for the labelled oligonucleotide were defined as those that could be successfully competed out by the inclusion of excess unlabelled oligonucleotide in the reaction.

When EMSAs were performed with the PU-1 or PEA3 sequences, no specific retarded complexes were observed with the I-1 and I-2 extracts (Figure 11.1): the low mobility band (labelled 'NS') was not efficiently competed by unlabelled oligonucleotides (data not shown). However, a single specific band (F1) was seen when F4-12 nuclear proteins were used with either of these oligonucleotides: the F2 and F3 bands are thought to be proteolytic degradation products of the F1 complex,

Figure 11.1: EMSAs using the PEA3 and PU-1 oligonucleotides.

Nuclear protein extract was isolated from I-1, I-2 and F4-12 cells and ^{32}P-end-labelled double-stranded PU-1 and PEA3 oligonucleotides prepared (see Methods and Materials). Binding reactions, using 500pg of labelled oligonucleotide and equal quantities of nuclear protein, and the non-denaturing polyacrylamide gel electrophoresis were performed as described in the Methods and Materials. Gels were dried and exposed to X-ray film for 12 hours. The **labelled oligonucleotide** used in each reaction is shown at the top of the figure, below which are the unlabelled **competitor** oligonucleotides incorporated into the binding reaction: (-) indicates no competitor, (+++) indicates 200-fold excess (100ng) of competitor. The **nuclear protein extract** used in each experiment is given under the competitors.
High mobility, non-specific binding, which is not competed by unlabelled oligonucleotide, is indicated by 'NS'. Specific complexes are named **F1**, **F2** and **F3**, and are discussed in the text. **Free oligonucleotide**, which has not been bound by any proteins in the extracts, is seen at the bottom of the autoradiograph.

Oligonucleotide sequences:

PEA3: GATCCTCGAGCAGGAAGTTCGAGATC
(Wasylyk *et al*, 1990).
PU-1: GATCCATAACCTCTGAAAGAGGAACTTGGTTAGGTGATC
(Klemsz *et al*, 1990).

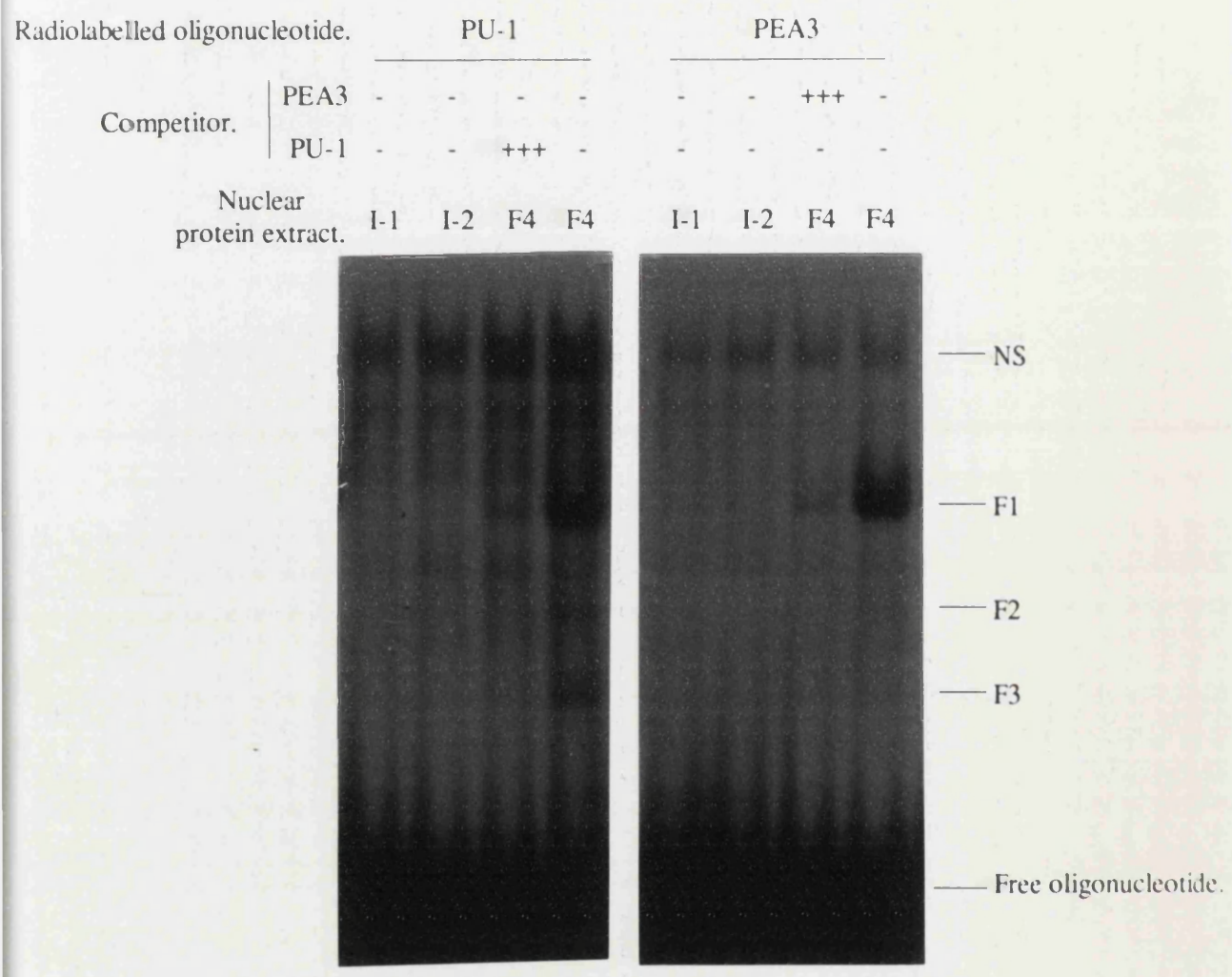

Radiolabelled oligonucleotide.
PU-1
PEA3
Competitor.
PEA3 - - - - - - +++ -
PU-1 - - +++ - - - - -
Nuclear protein extract.
I-1 I-2 F4 F4 I-1 I-2 F4 F4
NS
F1
F2
F3
Free oligonucleotide.

Figure 11.1.

since their intensity varies with the quality of the nuclear protein preparation (M. Grove and M. Plumb, personal communication). The F1 complex with the PU-1 sequence is almost certainly due to binding of Spi-1 and/or a closely-related protein since this sequence appears to preferentially bind these proteins (Klemsz *et al*, 1990; Ray *et al*, 1992): the comigration of the PEA3 band with this complex suggested that this too was probably due to the same protein(s) (Figure 11.1). This result shows that not only are Spi-1 and Spi-B absent from I-1 and I-2, but also c-ets-1, c-ets-2, elk-1 and PEA3 are probably not present, since there is no PEA3 binding activity in these cells. To support this hypothesis, a number of Northern blots were performed using RNA samples from the ELM system. The first probe used was a *Spi-1* cDNA containing the full coding region of the gene, generated by RT-PCR from spleen mRNA (see Materials and Methods), cloned into pUC19 and partially sequenced to verify its identity. Hybridising this sequence to a Northern blot demonstrated that *Spi-1* mRNA was undetectable in the *in vivo* passaged ELM tumour cells and all the cell lines derived from them (data not shown). However, more sensitive RT-PCR analysis of RNA samples suggested that a small amount of *Spi-1* mRNA was present in the ELM cell lines (data not shown): this probably represents the low level of expression apparent in normal erythroid progenitors (Galson *et al*, 1993). The F4-12 cell line, derived from a SFFV-induced erythroleukaemia, was shown to express a large amount of *Spi-1* mRNA as expected (data not shown). The lack of *c-ets-1* and *c-ets-2* gene expression was also shown by Northern analysis (data not shown), thus confirming the assumption made from the EMSA data with the PEA3 oligonucleotide.

Next, EMSAs were performed using the E74 oligonucleotide. All three of the cell lines under examination contained proteins that specifically bound to the E74 oligonucleotide. I-1 and I-2 gave one major band (A), two minor complexes (B1 and B2) and a band arising from non-specific binding to the oligonucleotide (C), whereas only the non-specific band C was observed using a mutated E74 oligonucleotide (E74(mut)) lacking the GGAA motif (Figure 11.2A and B). The only difference

Figure 11.2: EMSAs demonstrating the presence of E74 oligonucleotide binding activity in nuclear protein extract from erythroleukaemia cell lines.

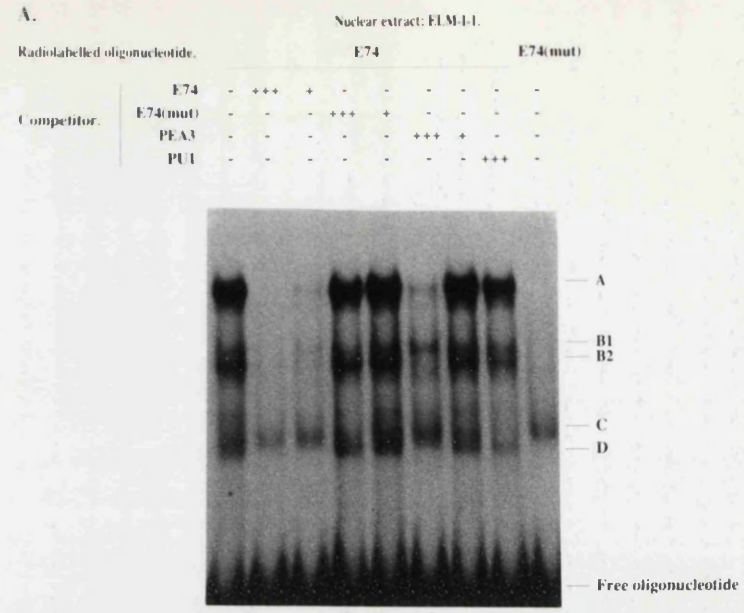
Nuclear protein extract was isolated from I-1, I-2 and F4-12 cells and ^{32}P-end-labelled double-stranded E74 oligonucleotides prepared (see Methods and Materials). Binding reactions, using 500pg of labelled oligonucleotide and equal quantities of nuclear protein, and the non-denaturing polyacrylamide gel electrophoresis were performed as described in the Methods and Materials. Gels were dried and exposed to X-ray film for 12 hours. The **labelled oligonucleotide** used in each reaction is shown at the top of each figure, below which are the unlabelled **competitor** oligonucleotides incorporated into the binding reaction: (-) indicates no competitor, (+) indicates 10-fold excess (5ng), (++) indicates 50-fold excess, (+++) indicates 200-fold excess (100ng).

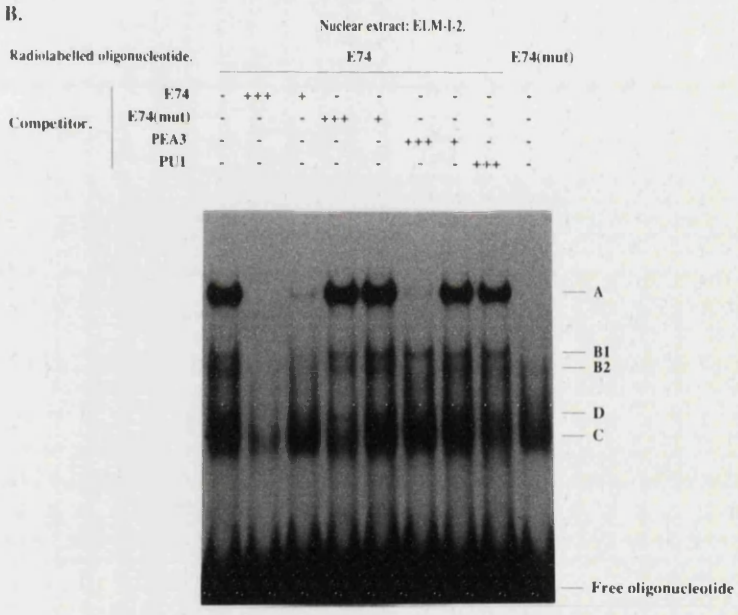
Panel **A**: Nuclear protein extract from ELM-I-1 cells.
Panel **B**: Nuclear protein extract from ELM-I-2 cells.
Panel **C**: Nuclear protein extract from Friend virus-induced erythroleukaemia F4-12 cells.

Low mobility, non-specific binding, which is not competed by unlabelled oligonucleotide, is indicated by the letter '**C**'. Specific complexes are named **A**, **B1**, **B2**, **D**, **F1**, **F2** and **F3**, and are discussed in the text. **Free oligonucleotide**, which has not been bound by any proteins in the extracts, is seen at the bottom of the autoradiograph.

Oligonucleotide sequences:

PEA3: GATCCTCGAGCAGGAAGTTCGAGATC
(Wasylyk *et al*, 1990).
PU-1: GATCCATAACCTCTGAAAGAGGAACTTGGTTAGGTGATC
(Klemsz *et al*, 1990).
E74: GATCTCTAGCTGAATAACCGGAAGTAACTCATCCTAGGATC
(Reddy and Rao, 1991).
E74(mut):GATCTCTAGCTGAATAACCCAAGTAACTCATCCTAGGATC
(Reddy and Rao, 1991).


A.
Nuclear extract: ELM-I-1.
Radiolabelled oligonucleotide.
E74
E74(mut)
Competitor.
E74 - +++ + - - - - - -
E74(mut) - - - +++ + - - - -
PEA3 - - - - - +++ + - -
PU1 - - - - - - - +++ -
A
B1
B2
C
D
Free oligonucleotide


B.
Nuclear extract: ELM-I-2.
Radiolabelled oligonucleotide.
E74
E74(mut)
Competitor.
E74 - +++ + - - - - - -
E74(mut) - - - +++ + - - - -
PEA3 - - - - - +++ + - -
PU1 - - - - - - - +++ -
A
B1
B2
D
C
Free oligonucleotide

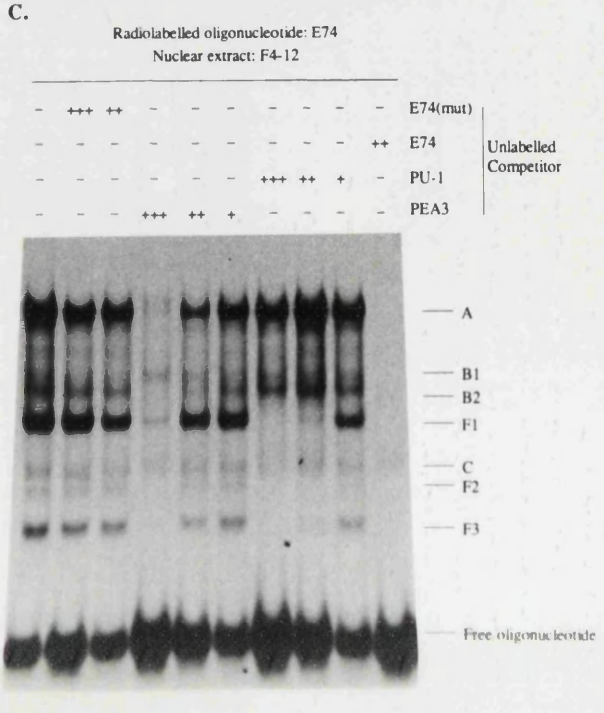

C.
Radiolabelled oligonucleotide: E74
Nuclear extract: F4-12
- +++ ++ - - - - - - - E74(mut)
- - - - - - - - - ++ E74
- - - - - - +++ ++ + - PU-1
- - - +++ ++ + - - - - PEA3
Unlabelled Competitor
A
B1
B2
F1
C
F2
F3
Free oligonucleotide

Figure 11.2.

between the two cell lines is the position of the weak band D. In I-1 it has a higher mobility than complex C: in I-2 its mobility is slightly less than C. However, band D may simply be due to proteolytic degradation of one of the lower mobility complexes. The retarded complexes were only competed efficiently by unlabelled E74, although a significant reduction in the formation of the A, B2 and D complexes was seen using 1,000-fold excess of the PEA3 oligonucleotide. The same pattern of bands was seen using F4-12 proteins (Figure 11.2C), although additional complexes were also observed which comigrate with the bands seen when F4-12 proteins were incubated with the labelled PEA3 and PU-1 oligonucleotides (Figure 11.1) and are competed with a low excess of unlabelled PU-1 oligonucleotide (Figure 11.2C): these, as before, are probably due to binding of Spi-1 and/or a closely-related protein. Recently, Zhang *et al* (1993) have demonstrated that Spi-1 is indeed able to bind to E74 when the protein is produced in bacteria fused to glutathione S transferase.

11.2: Fli-1 binds to the E74 sequence *in vitro*.

The EMSA results, confirmed by Northern analysis, showed that the *Spi-1*, *c-ets-1* and *c-ets-2* genes are not involved in the ELM system. However, I-1 and I-2 contain an E74 binding activity that does not bind well to the PU-1 and PEA3 sequences. The loss of binding to E74 when the GGAA motif is disrupted (E74(mut) sequence), and the weak competition observed with the PEA3 oligonucleotide is an indication that some, if not all, of the complexes formed with E74 are due to the binding of ets-related proteins present in the nuclear extracts. Currently, the only protein known to exhibit the DNA binding specificity observed in the I-1 and I-2 extracts is the human erg protein (Reddy and Rao, 1991). However, the extensive amino acid homology between erg and murine Fli-1, which is 98% over the putative DNA binding domain (Ben David *et al*, 1991), suggests that Fli-1 may exhibit the same specificity. Thus, experiments were performed to define the DNA binding specificity of Fli-1, using protein synthesised *in vitro*.

A cDNA fragment containing the Fli-1 coding region was generated by RT-PCR from murine spleen RNA, cloned into the pBluescriptII KS+ vector (to create a plasmid termed p*Fli*) and partially sequenced to verify its identity. *Fli-1* mRNA was then generated by transcription with T7 RNA polymerase from the p*Fli* plasmid linearised with *Sal* I. This was translated in a reticulocyte lysate system and aliquots used directly in EMSAs. From its homology with erg, it seemed unlikely the Fli-1 would bind to the PEA3, or the Spi-1-specific PU-1, oligonucleotides, and indeed, this proved to be the case (data not shown). However, using the E74 sequence, a complex was formed with Fli-1 (Figure 11.3) which comigrated with bands B1 and B2 observed in the I-1, I-2 and F4-12 EMSAs (Figure 11.2). The higher mobility complex, indicated by the lower arrow, was probably due to the binding of degraded or incompletely synthesised Fli-1. Both bands were competed out by unlabelled E74, and no binding was detected with the GGAA mutant oligonucleotide, E74(mut). Some oligonucleotide retardation was seen using a mock translation reaction that had not been primed with the *Fli-1* transcripts: this is presumably due to a low level of E74 binding activity present in the reticulocyte lysate. Thus, the Fli-1 and erg proteins produced *in vitro*, and nuclear proteins from the I-1 and I-2 cell lines, exhibit similar binding specificities. This result, in combination with the previously published EMSA data with the erg protein (Reddy and Rao, 1991), suggested that *erg* and *Fli-1* were likely candidate *ets* genes expressed in the ELM system. However, it must be remembered that the DNA binding specificity of a protein produced in an unphosphorylated state *in vitro*, in the absence of other transcription factors, could exhibit completely different properties in the context of a total nuclear protein extract prepared from growing cells. Indeed, a recurring theme with many ets proteins is that their ability to recognise certain DNA sequences is altered by phosphorylation and protein:protein interaction with non-ets-related transcription factors (discussed in the Introduction, sections 3.2 and 3.3).

Figure 11.3: EMSA showing binding of *in vitro*-translated Fli-1 to the E74 oligonucleotide.

The translation reaction and EMSA were carried out as described in Methods and Materials, using 500pg of the E74 or E74(mut) oligonucleotide as indicated at the top of the figure. 5µl (denoted by '+') or 10µl (denoted by '++') from the translation reaction containing **Fli-1 protein**, or from a **mock translation** reaction not primed with *Fli-1* mRNA, were included in the EMSA reaction where shown. The single reaction containing 50ng (100-fold excess) of **unlabelled E74** oligonucleotide is marked with a '+'. **Arrows** indicate the position of retarded complexes: the lower complex is probably due to binding of incompletely synthesised or degraded Fli-1 protein. **Free oligonucleotide**, which has not been bound by any proteins, is seen at the bottom of the autoradiograph.

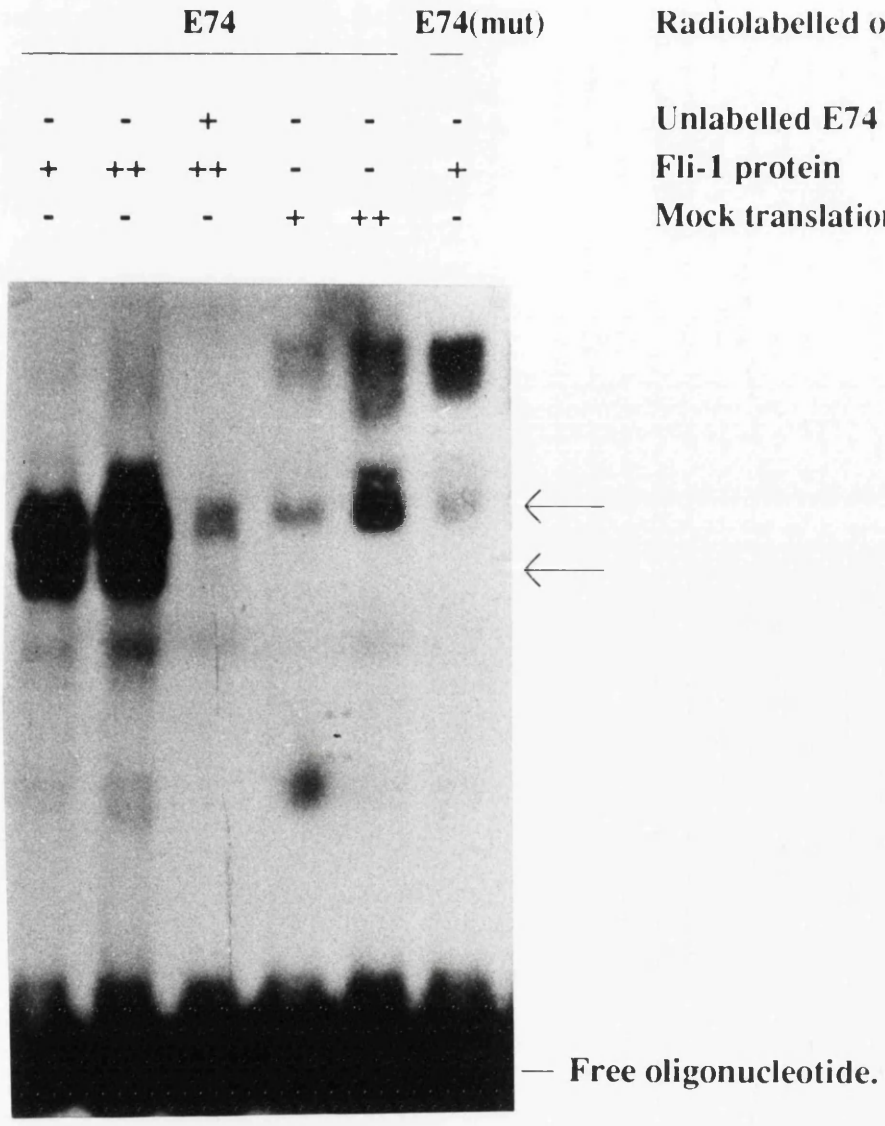

E74
E74(mut)
Radiolabelled oligonucleotide.
- - + - - -
Unlabelled E74 (100x excess).
+ ++ ++ - - +
Fli-1 protein
- - - + ++ -
Mock translation.
Free oligonucleotide.

Figure 11.3.

11.3: *Fli-1* is only expressed in the differentiation-resistant I-1 cell line.

To test whether the *erg* or *Fli-1* genes are in fact expressed in the ELM system, a series of Northern blots were performed. These experiments also allowed a comparison to be made of the various phenotypically-distinct cell lines of the ELM system. First, the insert from the p*Fli* plasmid was used as a probe. The reduction in *Fli-1* mRNA in mouse spleen upon stimulation of erythropoiesis by induction of anaemia with phenylhydrazine (Methods and Materials), suggests that *Fli-1* is only expressed at low levels, if at all, in developing erythroid cells (Figure 11.4). Only a very small quantity of *Fli-1* mRNA was found to be present in leukaemic spleens containing ELM cells which had been continually passaged *in vivo* since the development of the original malignancy. This is consistent with it being expressed solely in a small number of contaminating normal spleen cells. An RNA sample from a very early passage of the cells was also extremely low in *Fli-1* transcripts, implying that deregulated expression of this gene was not involved in the evolution of this erythroleukaemia. ELM-D and D#6 cells, growing in contact with stromal cells, also expressed the gene to a very low level (data not shown) which was thought to be due to the presence of contaminating stromal cell RNA in the RNA sample. To confirm this, D#6 cells were separated from the MS-5 cells by centrifugal elutriation, and RNA extracted (see Methods and Materials): *Fli-1* mRNA was undetectable in this pure population of D#6 cells (Figure 11.4). Interestingly, *Fli-1* mRNA was absent from all the fifteen stromal cell-independent cell lines tested, with the exception of I-1, where it was highly abundant (Figure 11.4 and data not shown). Since this cell line is unable to differentiate in response to epo, this property may be the result of *Fli-1* over-expression. However, the lack of *Fli-1* mRNA in the fourteen other stromal cell-independent cell lines, indicates that expression of this gene is not a prerequisite for stromal cell-independent growth.

The presence of *Fli-1* mRNA in the I-1 cells, and the results obtained using *in vitro*-translated Fli-1, raised the possibility that some of the E74 binding activity in the nuclear extract prepared from this cell line was due to binding of the Fli-1 protein.

Figure 11.4: Northern blots showing that *Fli-1* gene expression is restricted in the ELM system to the differentiation-resistant cell line, ELM-I-1.

20μg of total cellular RNA was electrophoresed, blotted and hybridised to a ^{32}P-labelled full-length *Fli-1* cDNA probe (Methods and Materials). Exposure time: 12 hours. Molecular weight markers are given, in kb, between the autoradiographs. The blot was stripped and reprobed with an *actin* cDNA to assess the relative levels of RNA loading.

Tissues and cell lines examined were:
I-1, I-2, I-5 and **I-6**:- Stromal cell-independent clones I-1, I-2, I-5 and I-6.
D#6 elutriated at 0.3ml/sec:- Pure ELM-D#6 cells prepared by centrifugal elutriation from a freshly-harvested ELM-D#6/MS-5 coculture (Methods and Materials).
MS-5:- Stromal cell line, MS-5.
IVP ELM:- *In vivo* passaged ELM tumour cells:
Late passage (5 passages since the evolution of the ELM tumour).
Early passage (1 passage since the evolution of the ELM tumour).
See Figure 5.1 in the Introduction.
Spleen:- Normal C3H mouse spleen.
Anaemic spleen:- Erythropoietically-active spleen from a C3H mouse which had been treated with phenylhydrazine to induce anaemia (see Methods and Materials).

The IVP ELM (late passage) sample was included on both blots to permit comparisons to be made between the two autoradiographs.

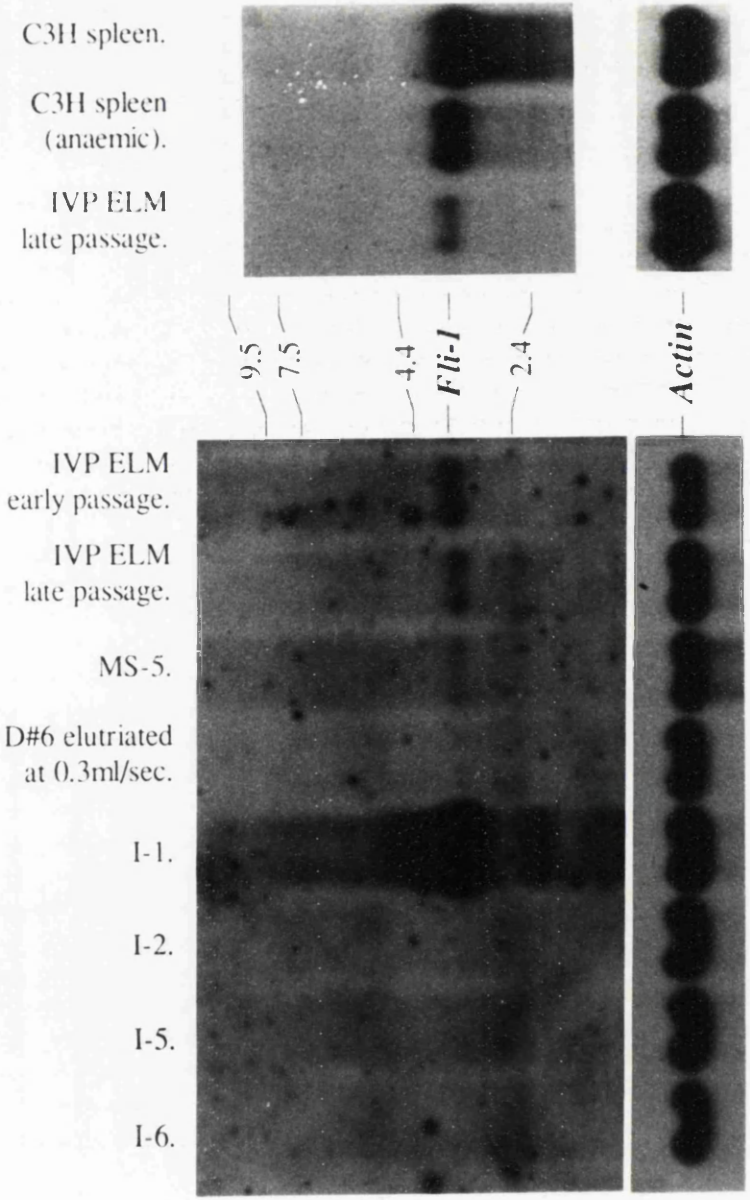

C3H spleen.
C3H spleen (anaemic).
IVP ELM late passage.
9.5
7.5
4.4
Fli-1
2.4
Actin
IVP ELM early passage.
IVP ELM late passage.
MS-5.
D#6 elutriated at 0.3ml/sec.
I-1.
I-2.
I-5.
I-6.

Figure 11.4.

Furthermore, the F4-12 cell line also expresses the *Fli-1* gene at a low level (data not shown), and generated a banding pattern with the E74 sequence that was very similar to the I-1 cell line. However, the lack of *Fli-1* gene expression in I-2 cells questions this assumption, since a virtually identical banding pattern is observed between the E74 sequence and I-2 nuclear extract. Bearing in mind the similar size and DNA binding specificities of Fli-1 and erg, it seemed worthwhile to test whether I-2 cells expressed the murine homologue of the human erg protein which may cause the similarity observed in the EMSAs.

11.4: Correlation between stromal cell-independent growth and expression of the *erg* gene.

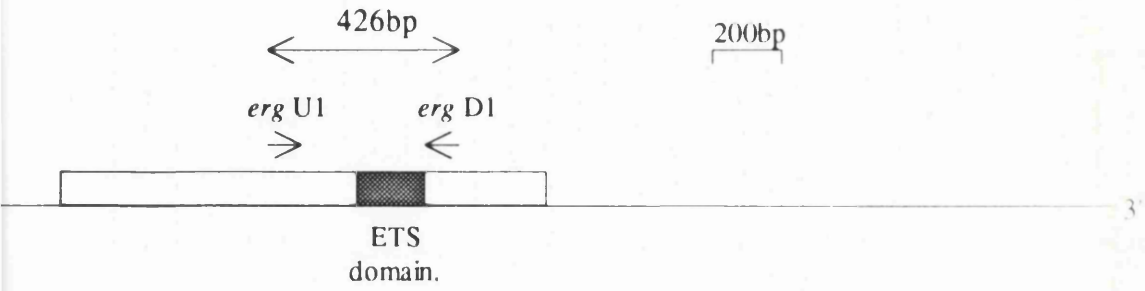
11.4.1: Cloning part of the murine *erg* cDNA.

Rather than use a human *erg* DNA probe which may not be able to cross-hybridise to the putative murine homologue, a cDNA fragment spanning the *ets* domain of murine *erg* was cloned. A single-stranded DNA template was generated by reverse transcription from various RNA samples primed with a poly-T oligonucleotide. This was then subjected to PCR using two primers designed from the human *erg* sequence (Rao *et al*, 1987) from regions that show strong cross-species conservation between other *ets*-related genes (Figure 11.5A). Three fragments were produced from mouse spleen RNA: one had the expected size of 426bp, but a larger (~500bp) and a smaller (~340bp) product were also generated (Figure 11.5B). The same bands were synthesised from I-2 RNA, but were absent from D#6 and F4-12 samples. It must be remembered, however, that since the PCR primers used were designed from the human *erg* sequence, the absence of an amplified product from mouse cell RNA does not necessarily mean that the mouse homologue is not expressed in these cells: it may be expressed at a level lower than I-2 which prevents efficient amplification. Incidentally, the 426bp and 500bp fragments were also amplified from RNA from the human T cell line, Jurkat, which is known to express *erg* (Rao *et al*, 1987) and was used here as a positive control: the 340bp product was

Figure 11.5: Cross-species RT-PCR analysis of *erg* expression in selected murine cells.

A: Human *erg* cDNA structure (Rao *et al*, 1987) showing the position of the ***erg* U1** and ***erg* D1** primers used in the RT-PCR reaction shown in panel B. The sequence of the primers is shown in table 6.2 in the Methods and Materials. The **ETS domain** is represented as a shaded box.

B: Ethidium bromide stained gel, photographed under UV illumination, of RT-PCR reactions for *erg*. RT-PCR was performed as described in the Methods and Materials, using the primers shown in panel A; half the reaction was electrophoresed on a 1% (w/v) agarose gel. Molecular weight DNA markers are shown to the left of the gel photograph. The presence (+) or absence (-) of reverse transcriptase (**RT**) during the cDNA production is indicated at the top of the gel.
Cell lines and tissues tested were Jurkat cells, a human T cell line (used as a positive control), Friend virus-induced erythroleukaemia F4-12 cells, ELM-I-2 cells, ELM-D#6 cells on MS-5 stroma (ratio of 6 D#6 per MS-5), and C3H spleen.


426bp
200bp
erg U1
erg D1
3'
ETS
domain.

B.

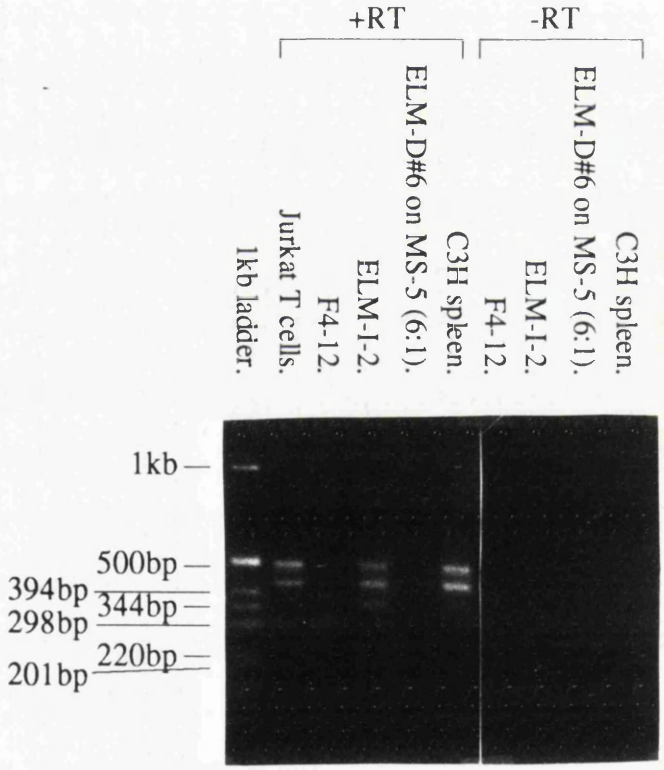

+RT
-RT
1kb ladder.
Jurkat T cells.
F4-12.
ELM-I-2.
ELM-D#6 on MS-5 (6:1).
C3H spleen.
F4-12.
ELM-I-2.
ELM-D#6 on MS-5 (6:1).
C3H spleen.
1kb
500bp
394bp
344bp
298bp
220bp
201bp

Figure 11.5.

absent. Control reactions in which the reverse transcriptase had been omitted from the cDNA production step, were all negative.

Attempts were made to clone the three products using the TA cloning kit (see Materials and Methods), but this was only successful for the 500bp and 426bp products, generating the p500*erg* and p426*erg* plasmids. The insert of p426*erg* was sequenced, translated, and aligned with human erg and murine Fli-1 amino acid sequences (Figure 11.6). Unsurprisingly, like other ets proteins, erg is highly conserved between species, with only four amino acids variant in the region cloned; it is also closely related to Fli-1, as previously described for the human homologues (Watson *et al*, 1992). Interestingly, the differences between Fli-1 and erg found in the putative DNA binding domain reside in a cluster of serine residues, possible targets for covalent modifications. The 500bp and 426bp inserts were identical, but 500*erg* contained sequence coding for a further 24 amino acids amino-terminal to the ets domain, and probably arises as a result of alternative splicing of the *erg* mRNA (Figure 11.6). This region of erg and Fli-1 adjacent to the ets domain is the least conserved part of the protein and may be involved in altering the DNA binding activity and specificity of the two proteins *in vivo* by mediating protein-protein interactions, or by acting as targets for specific post-translational modifications. The equivalent portion of several other ets-related proteins is believed to behave in this manner (c-ets-1 (Wasylyk *et al*, 1992); Spi-1 (Pongubala *et al*, 1992, 1993); elk-1 (Janknecht and Nordheim, 1992; Rao and Reddy, 1992b); SAP-1 (Dalton and Treisman, 1992); GABP-α (Thompson *et al*, 1991)).

11.4.2: Expression of the *erg* gene in the ELM system.

The *erg* cDNA fragment in the p426*erg* plasmid was excised, labelled with ^{32}P and hybridised to Northern blots of RNA samples from the cells of the ELM system (Figure 11.7). As with *Fli-1*, the reduction of *erg* mRNA in mouse spleen upon stimulation of erythropoiesis by induction of anaemia with phenylhydrazine (Methods and Materials), suggests that *erg* is only expressed at low levels, if at all, in

Figure 11.6: Amino acid sequence comparison of part of the human (h) and murine (m) erg and Fli-1 proteins.

Conserved amino acids, relative to the human erg sequence, are represented by a dash (-); absent amino acids are indicated by a dot (.). The ETS domain, the putative DNA binding portion of the protein, is contained between the two brackets ([]), with the conserved tryptophan residues highlighted with an asterisk (*).
The sequence of the murine erg was derived by translating the nucleotide sequence generated from the insert of the p426*erg* plasmid, although the insert of the p500*erg* plasmid was identical except that a region encoding a further 24 amino acids (shown at the base of the figure) is present at the point marked '**alt**' (see text for the derivation of the two *erg* plasmids). The ***erg* U1** and ***erg* D1** primers, used in the PCR reactions to generate murine erg cDNA fragments, are shown.

The sequence and amino acid numbering of the other proteins shown here was derived from the following references:

Rao *et al*, 1987 (Human erg).
Watson *et al*, 1992 (Human Fli-1).
Ben-David *et al*, 1991 (Murine Fli-1).

```
erg (h)        DKALQNSPRLMHARNT   DLPYEPPRRSAWTGHGHPTPQSKAAQPS 259

426erg (m)     ergU1 primer____alt---------------S-L----------

Fli-1 (h)      TSHTDQ-S--..SVKE   -PS-DSV--G--GNNMNSGLNKSPPLGG 246
Fli-1 (m)      TSHTDQ-S--..NVKE   -PS-DSV--G--NNNMNSGLNKSPLLGG 246


                                              [       *
erg (h)        PSTVPKTEDQRPQLDPYQILGPTSSRLANPGSGQIQLWQFLLELLS 305

426erg (m)     --A-----------------------------------------

Fli-1 (h)      AQ-IS-NTE----P-------------------------------- 292
Fli-1 (m)      SQ-MG-NTE----P-------------------------------- 292


                        *                    *
erg (h)        DSSNSSCITWEGTNGEFKMTDPDEVARRWGERKSKPNMNYDKLSRA 351

426erg (m)     -----N-----------------------------------------

Fli-1 (h)      --A-A------------------------------------------ 338
Fli-1 (m)      --A-A------------------------------------------ 338


                                       ]
erg (h)        LRYYYDKNIMTKVHGKRYAYKFDFHG 377

426erg (m)     __ergD1 primer

Fli-1 (h)      -------------------------- 364
Fli-1 (m)      -------------------------- 364


  Alternatively spliced into 'alt' in 500erg:

               GGAAFIYPNTSVYPGATQRITTRP
```

FIGURE 11.6.

developing erythroid cells. More importantly, these experiments showed the *erg* mRNA to be barely detectable in the *in vivo* passaged tumour cells from either early or late passages, or in the D#6 cell line and two other stromal cell-dependent clones (D#2 and D#3) (Figure 11.7A). However, in contrast, it was relatively abundant in thirteen of the fourteen stromal cell-independent cell lines tested (Figure 11.7A and B). Once differences in the loading have been normalised using the actin control, scanning densitometry revealed that these cell lines express *erg* at levels on average approximately 20-fold higher (ranging between 4- and 48-fold) than the D#6 cells from which most of them were derived. Thus, there appeared to be a strong correlation between increased expression of the *erg* gene and stromal cell-independent growth in the ELM system.

In interpreting this experiment, account must be taken of the level of MS-5 contamination in the coculture. Before harvesting the D#6/MS-5 coculture, it was estimated that there were six leukaemic cells per stromal cell. This figure was generated by counting the number of D#6 cells adhered to approximately twenty stromal cells from a representative patch of all the tissue culture flasks from which RNA was isolated. Contamination of roughly 15%, was confirmed by the *p53* PCR from genomic DNA, and subsequent *Dra* III cleavage, shown in Figure 10.6. However, it remained a possibility that MS-5 cells contribute a disproportionate quantity of RNA when harvested with D#6 cells.

One way to test for this is to probe the Northern blots with either an MS-5- or a D#6-specific cDNA probe, such as *SCF* or *c-kit*, respectively, but these experiments may be subject to error since coculture of the two cell types may alter the expression of any such markers. Indeed, this point was implied from earlier work examining *SCF* expression which demonstrated that a D#6/MS-5 culture with a low ratio of leukaemic to stromal cells, expresses this growth factor gene at a level considerably greater than MS-5 cells alone, indicating a possible up-regulation of this gene as a consequence of coculture (data not shown). Instead, various ratios of freshly-harvested I-1 and MS-5 cells were mixed and RNA immediately extracted. This

Figure 11.7: Northern blots demonstrating that expression of the *erg* gene is restricted to the stromal cell-independent clones within the ELM system.

20μg of total cellular RNA was electrophoresed, blotted and hybridised to a ^{32}P-labelled *erg* cDNA fragment excised from the p500*erg* plasmid (Methods and Materials). Exposure time for both blots was 6 days. Molecular weight markers are given, in kb, to the right of the autoradiographs. The blot was stripped and reprobed with an *actin* cDNA to assess the relative levels of RNA loading: exposure time was 6 hours. The intensity of all the *actin* and *erg* bands on both blots was determined by scanning densitometry. The figures for the *erg* bands were then modified to take into account the differences in RNA loading determined by the *actin* bands, setting the D#6 on MS-5 (6:1) sample to 1.0: the adjusted values are given below the autoradiographs.

Tissues and cell lines examined were:

In panel A:
D#3 on MS-5 (5:1):- ELM-D#3 cells growing on live MS-5 cells (5 ELM-D#3 cells per MS-5 cell, as determined by visually counting the cells prior to RNA extraction).
D#2 on MS-5 (6:1):- ELM-D#2 cells growing on live MS-5 cells (6 ELM-D#2 cells per MS-5 cell, as determined by visually counting the cells prior to RNA extraction).
I-1, I-2, I-5 and **I-6**:- Stromal cell-independent clones I-1, I-2, I-5 and I-6.
D#6 on MS-5 (6:1):- ELM-D#6 cells growing on live MS-5 cells (6 ELM-D#6 cells per MS-5 cell, as determined by visually counting the cells prior to RNA extraction).
MS-5:- Stromal cell line, MS-5.
IVP ELM:- *In vivo* passaged ELM tumour cells:
Late passage (5 passages since the evolution of the ELM tumour).
Early passage (1 passage since the evolution of the ELM tumour).
See Figure 5.1 in the Introduction.
C3H spleen:- Normal C3H mouse spleen.
C3H spleen (anaemic):- Erythropoietically-active spleen from a C3H mouse which had been treated with phenylhydrazine to induce anaemia (see Methods and Materials).

In panel B:
D#6 on MS-5 (6:1):- ELM-D#6 cells growing on live MS-5 cells (6 ELM-D#6 cells per MS-5 cell, as determined by visually counting the cells prior to RNA extraction).
I-1 and **I-2**:- Stromal cell-independent clones I-1 and I-2.
Ten **ELM-I lines derived from D#6 in the presence of GM-CSF**. These were cloned from ELM-D#6 in the presence of GM-CSF, but subsequent analysis demonstrated that they were not dependent on GM-CSF for long-term survival without stromal cell contact (see Figure 5.1, and text in section 5.4, in the Introduction).

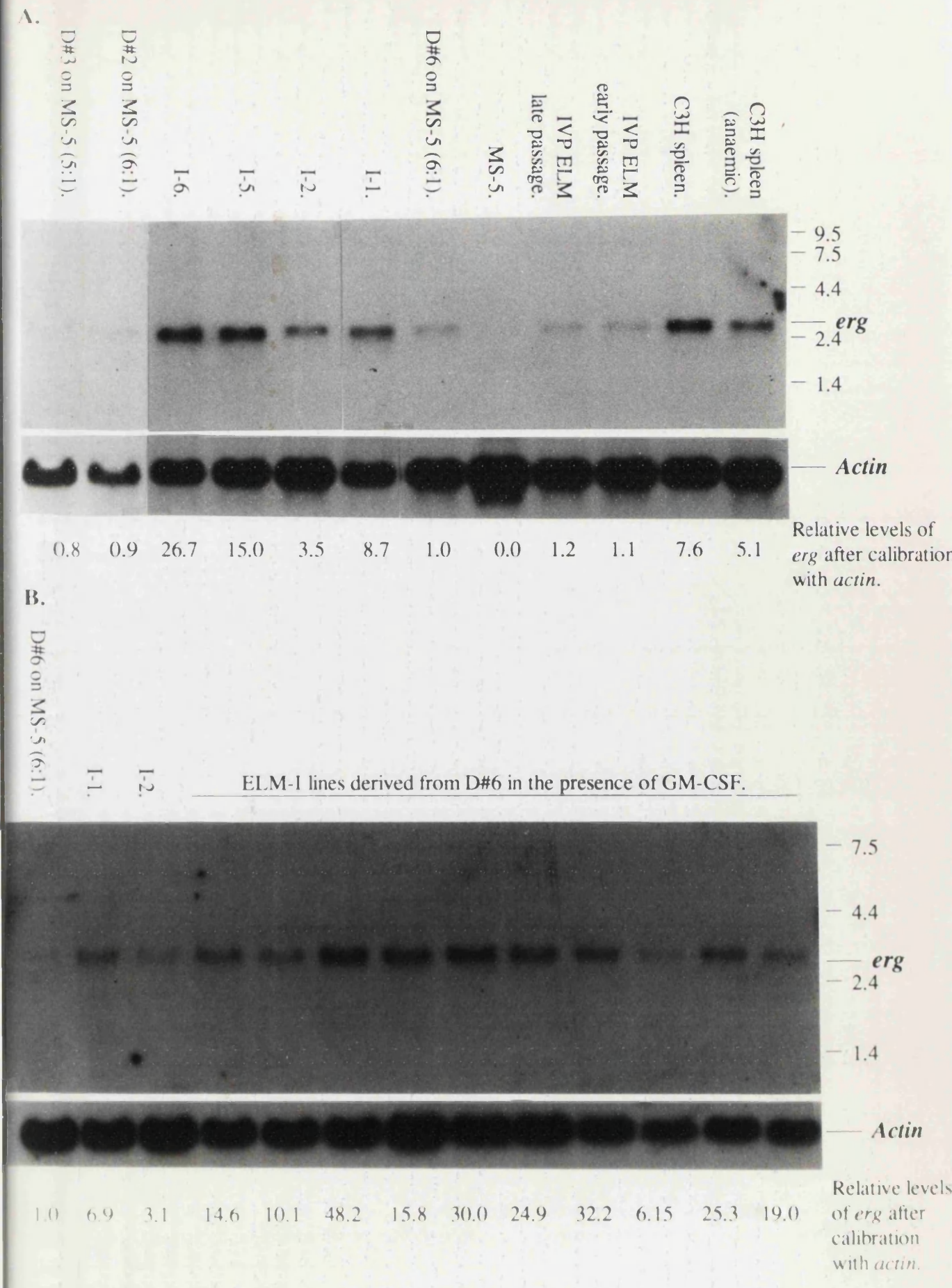

A.
D#3 on MS-5 (5:1).
D#2 on MS-5 (6:1).
I-6.
I-5.
I-2.
I-1.
D#6 on MS-5 (6:1).
MS-5.
IVP ELM late passage.
IVP ELM early passage.
C3H spleen.
C3H spleen (anaemic).
9.5
7.5
4.4
erg
2.4
1.4
Actin
0.8 0.9 26.7 15.0 3.5 8.7 1.0 0.0 1.2 1.1 7.6 5.1
Relative levels of erg after calibration with actin.
B.
D#6 on MS-5 (6:1).
I-1.
I-2.
ELM-I lines derived from D#6 in the presence of GM-CSF.
7.5
4.4
erg
2.4
1.4
Actin
1.0 6.9 3.1 14.6 10.1 48.2 15.8 30.0 24.9 32.2 6.15 25.3 19.0
Relative levels of erg after calibration with actin.

Figure 11.7.

avoids interactions between the two cell types, thus preventing contact-dependent alterations in gene expression. For comparison, samples containing different ratios of I-1/MS-5 RNA were also created by mixing the appropriate amounts of RNA from pure I-1 and MS-5 preparations. A Northern blot was generated using all these samples, plus samples of pure MS-5 and I-1 RNA as controls, and probed with a full-length *Fli-1* cDNA (Figure 11.8). After exposure to X-ray film, the filter was stripped of the *Fli-1* probe and hybridised to an *actin* cDNA to assess the relative loading between the samples. Both autoradiographs were then subjected to scanning densitometry and the levels of *Fli-1* adjusted relative to the pure I-1 sample (given as 1.0 in Figure 11.8). By a direct comparison between the cell and RNA mixes, it was concluded that MS-5 cells may contain slightly more RNA than ELM cells. However, the effect of dilution of D#6 by MS-5 cells insufficient to account for the differences in *erg* mRNA level observed between a D#6/MS-5 coculture and the stromal cell-independent clones, which can be up to 48-fold higher (Figure 11.7).

11.5: Up-regulation of the *erg* gene in D#6 cells upon separation from MS-5 cells.

To determine whether this up-regulation of *erg* mRNA is due to adaptation to loss of stroma cell contact, or only found in truly stromal cell-independent mutants, pure populations of D#6 cells were obtained, either by taking D#6 cells that had detached from the stromal cells in a high density coculture, or by cleanly separating attached D#6 cells from MS-5 cells by trypsinisation, followed by elutriation and then short-term culture (~2-3hrs) (see Methods and Materials). Subsequent Northern blot analysis of RNA samples extracted from these cells revealed 4-9 fold higher levels of *erg* mRNA in the pure populations of D#6 cells compared to the 6:1 D#6/MS-5 cocultures. This compares with the 14-40 fold higher levels in the three ELM-I mutants analysed at the same time (Figure 11.9). Further experiments confirmed that the pure populations of D#6 cells were not capable of long-term growth in the absence of MS-5 stromal cells, but died out within 1-2 weeks (data not shown). As before, these differences in the level of gene expression are too large to be accounted

Figure 11.8: Dilution of *Fli-1* mRNA by MS-5 RNA.

The indicated ratios of freshly-harvested I-1 and MS-5 cells were mixed and RNA immediately extracted to avoid an interaction between the two cell types, thus preventing contact-dependent alterations in gene expression. Samples containing the same ratios of RNA were also created by mixing the appropriate amounts of RNA from pure I-1 and MS-5 preparations. A Northern blot was generated (see Methods and Materials) using 20μg of these mixes, plus a 20μg sample of pure MS-5 and I-1 RNA. This was probed with a full-length *Fli-1* cDNA. Exposure time: 16 hours. Molecular weight markers are given, in kb, to the right of the autoradiograph. The filter was stripped of the *Fli-1* probe and hybridised to an *actin* cDNA to assess the relative loading between the samples. Exposure time: 3 hours. The intensity of all the *actin* and *Fli-1* bands the autoradiograph was determined by scanning densitometry. The figures for the *Fli-1* bands were then modified to take into account the differences in RNA loading determined by the *actin* bands, setting the pure I-1 sample to 1.0: the adjusted values are given below the autoradiograph.

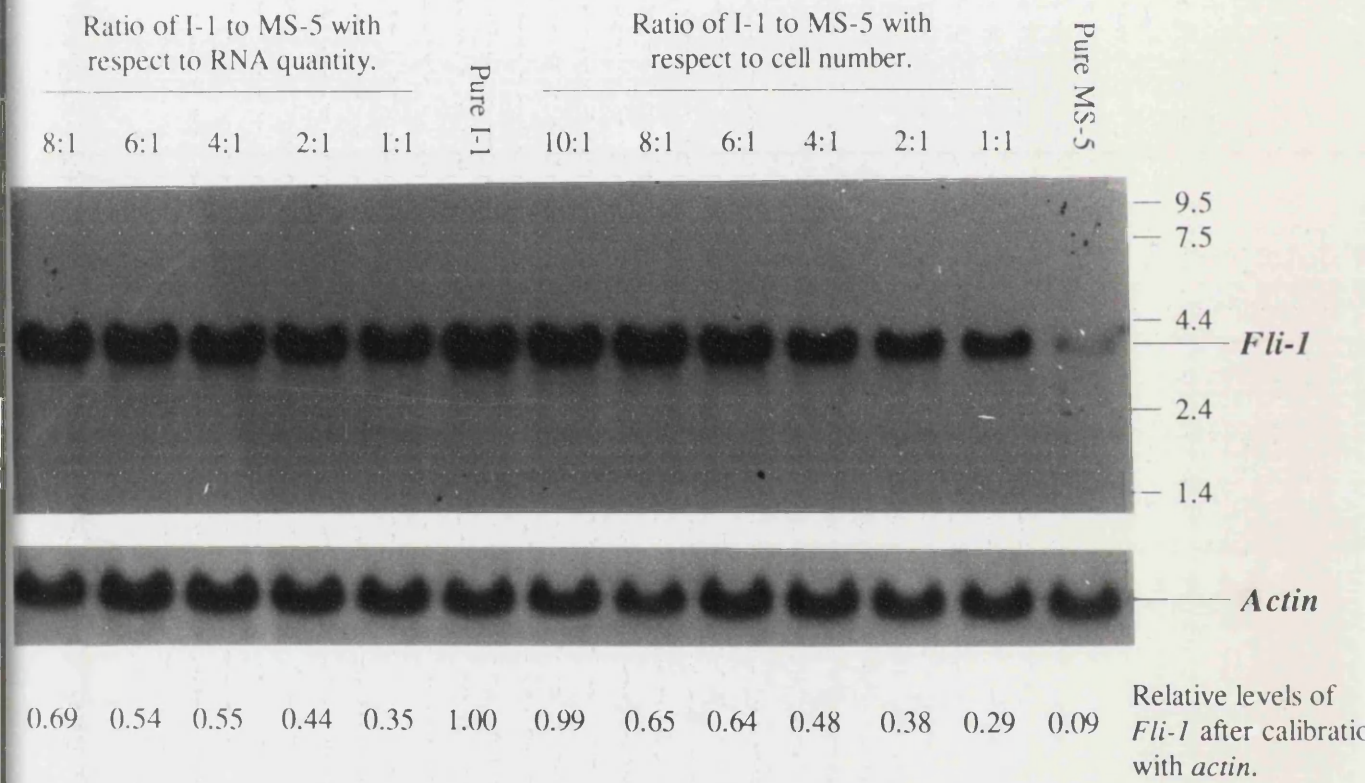

Ratio of I-1 to MS-5 with respect to RNA quantity.
Pure I-1
Ratio of I-1 to MS-5 with respect to cell number.
Pure MS-5
8:1
6:1
4:1
2:1
1:1
10:1
8:1
6:1
4:1
2:1
1:1
9.5
7.5
4.4
2.4
1.4
Fli-1
Actin
0.69
0.54
0.55
0.44
0.35
1.00
0.99
0.65
0.64
0.48
0.38
0.29
0.09
Relative levels of Fli-1 after calibratio with actin.

Figure 11.8.

Figure 11.9: Separating ELM-D#6 cells from stromal cells induces expression of the *erg* gene.

20μg of total cellular RNA was electrophoresed, blotted and hybridised to a ^{32}P-labelled *erg* cDNA fragment excised from the p500*erg* plasmid (Methods and Materials). Exposure time: 6 days. Molecular weight markers are given, in kb, to the right of the autoradiographs. The blot was stripped and reprobed with an *actin* cDNA to assess the relative levels of RNA loading: exposure time was 6 hours. The intensity of all the *actin* and *erg* bands was determined by scanning densitometry. The figures for the *erg* bands were then modified to take into account the differences in RNA loading determined by the *actin* bands, setting the D#6 on MS-5 (6:1) sample to 1.0: the adjusted values are given below the autoradiograph.

Cell lines examined:
One of the **ELM-I lines derived from D#6 in the presence of GM-CSF** (shown previously in Figure 11.7).
I-5 and **I-6**:- Stromal cell-independent clones I-5 and I-6.
D#6 elutriated at 0.3ml/sec:- Pure ELM-D#6 cells prepared by centrifugal elutriation from a freshly-harvested ELM-D#6/MS-5 coculture, followed by a brief period of culture (2-3 hours) (see Methods and Materials, section 6.6.1, for a detailed description of procedure).
D#6 suspension cells:- ELM-D#6 cells taken from the medium of a dense ELM-D#6/MS-5 coculture.
MS-5:- Stromal cell line, MS-5.

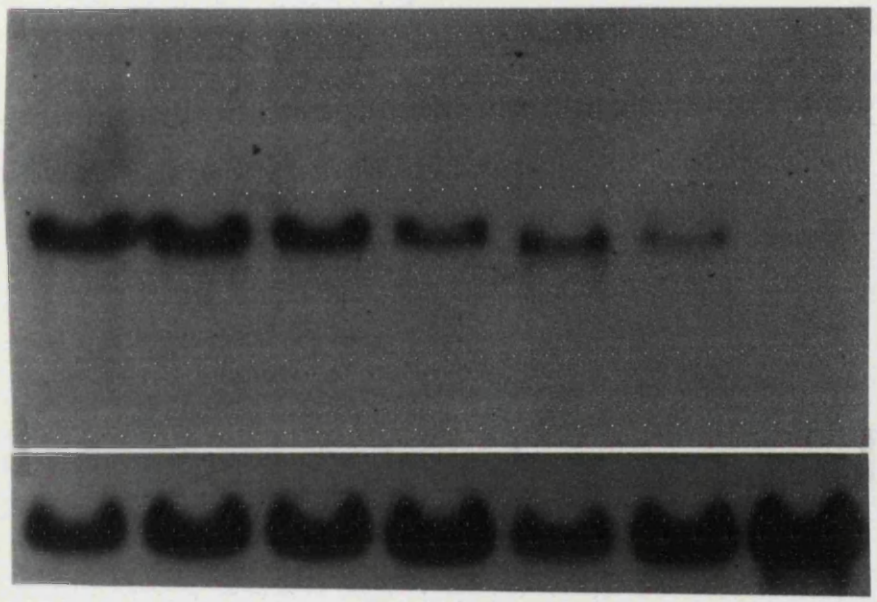

ELM-I clone derived in presence of GM-CSF.
I-6.
I-5.
D#6 elutriated at 0.3ml/sec.
D#6 suspension cells.
D#6 on MS-5 (6:1).
MS-5.
9.5
7.5
4.4
erg
2.4
1.4
Actin
42.6
20.2
14.2
4.4
9.1
1.0
0.0
Relative levels of erg after calibration with actin.

Figure 11.9.

for by dilution of the *erg* mRNA level by the 15% MS-5 stroma cells in the D#6/MS-5 coculture. These results, therefore, strongly suggest that *erg* mRNA is up-regulated in response to loss of stroma cell contact, but that this is not sufficient *per se* to allow the long-term stroma cell-independent growth characteristic of the ELM-I mutants.

11.6: Effect of MS-5 contact on the level of *erg* gene expression in I-2 and I-5 cells.

D#6 cells survive for several days in the absence of stromal cell contact and during this period are capable of limited proliferation (Itoh *et al*, 1993). It was postulated from the results described above that this may, at least in part, be due to the expression of the *erg* gene in the D#6 cells. Moreover, it is therefore possible that constitutive expression of the *erg* gene could indefinitely extend this period of stromal cell-independent growth, in other words generating ELM-I clones. To examine whether *erg* expression in the ELM-I clones is no longer subject to the same controls as that observed in D#6 cells, the following experiment was devised to assess the effect of stromal contact on the expression of the gene in the I-2 and I-5 cell lines. MS-5 cells were seeded at 50% and 80% confluence in fresh medium, a six-fold excess of I-2 or I-5 cells was added, and the coculture incubated for three days at 37°C. Both the ELM-I cell lines used, like the ELM-D cells, adhered strongly to the MS-5 monolayer (data not shown). ELM-I cells remaining in suspension were discarded prior to harvesting the cells, the ratio of ELM-I to MS-5 cells was determined and total cellular RNA extracted. As controls, parallel cultures of MS-5 cells alone were grown for three days, harvested, mixed with the same ratio of I-2 or I-5 cells as determined for the cocultures, and the RNA immediately extracted. Fresh samples of pure I-2 and I-5 RNA were also prepared. A Northern blot was generated with all these preparations, and probed first with the *erg* cDNA fragment excised from the p500*erg* plasmid, and then with an *actin* cDNA fragment to assess the relative loading of the samples (Figure 11.10). This blot showed that culture with MS-5 cells had no effect on the level of *erg* mRNA in either the I-2 or the I-5 cell

Figure 11.10: Northern blot demonstrating that contact between MS-5 cells and the stromal cell-independent cell lines I-2 and I-5, has no effect on *erg* gene expression.

I-2 and I-5 cells were harvested and incubated to a six-fold excess in contact with a 50% or 80% confluent MS-5 monolayer for three days: RNA was then extracted. The same mixes were prepared using pure MS-5 cells and I-2 or I-5 cells, but RNA was extracted immediately without incubation, and before the cells became adhered to one another. RNA was also prepared from pure I-5 and I-2 cells. 20μg of each RNA sample was electrophoresed, blotted and hybridised to a ^{32}P-labelled *erg* cDNA fragment excised from the p500*erg* plasmid (Methods and Materials). Exposure time: 6 days. Molecular weight markers are given, in kb, to the right of the autoradiographs. The blot was stripped and reprobed with an *actin* cDNA to assess the relative levels of RNA loading: exposure time was 6 hours. The intensity of all the *actin* and *erg* bands was determined by scanning densitometry. The figures for the *erg* bands were then modified to take into account the differences in RNA loading determined by the *actin* bands, setting the I-2 only sample (lane furthest right) to 1.0: the adjusted values are given below the autoradiograph.

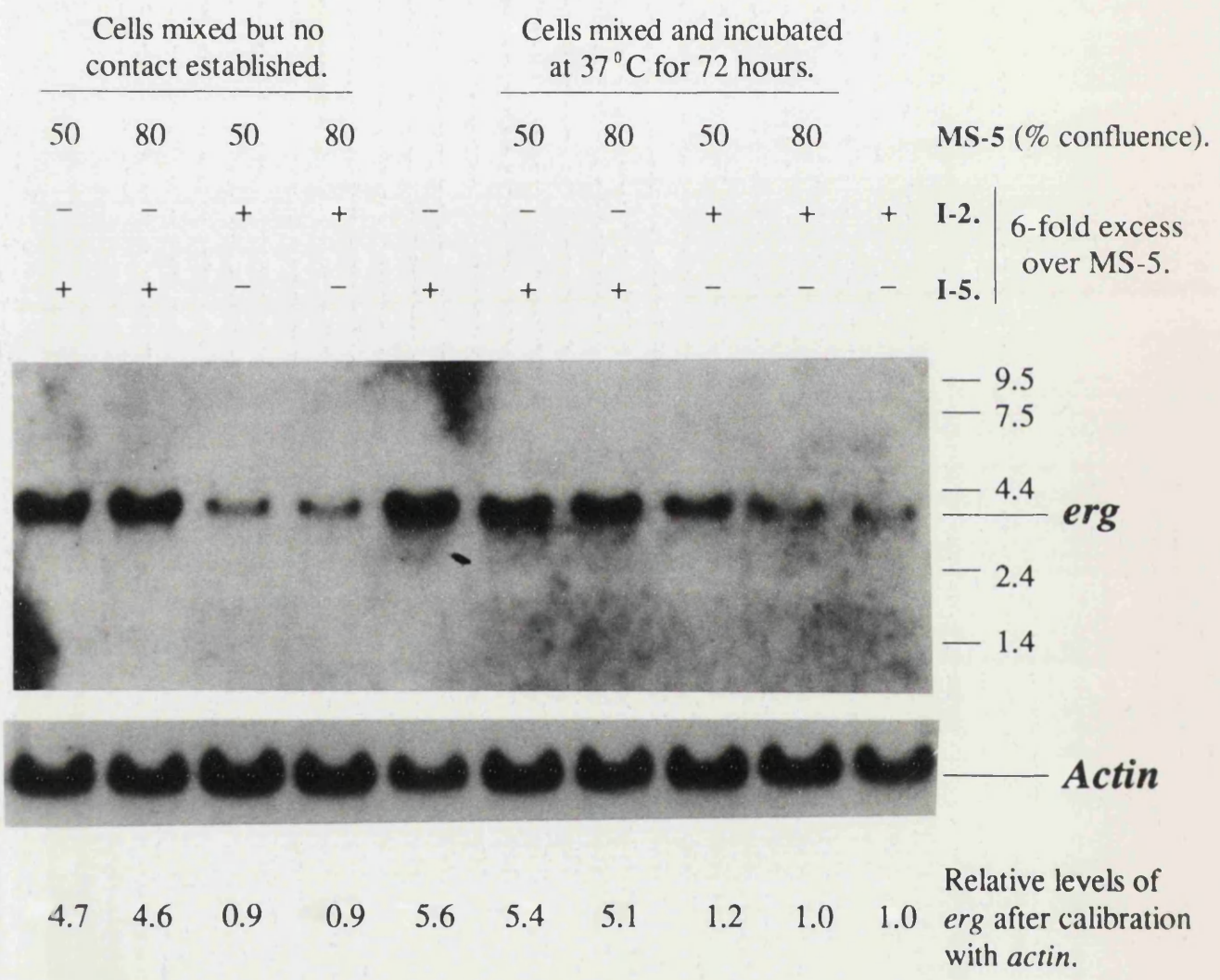

Cells mixed but no contact established.
Cells mixed and incubated at 37 °C for 72 hours.
50 80 50 80 50 80 50 80
MS-5 (% confluence).
− − + + − − − + + +
I-2.
6-fold excess over MS-5.
+ + − − + + + − − −
I-5.
9.5
7.5
4.4
erg
2.4
1.4
Actin
4.7 4.6 0.9 0.9 5.6 5.4 5.1 1.2 1.0 1.0
Relative levels of erg after calibration with actin.

Figure 11.10.

lines, which remained well above the level observed in a coculture of MS-5s with either D#2, D#3 or D#6 (Figure 11.7A).

These observations suggest that the mutation(s) responsible for the generation of stromal cell-independent growth and long-term viability contrive to prevent normal regulation of the *erg* gene in response to stromal cell contact. Although yet to be proven, this deregulated *erg* expression may be a crucial step in the development of stromal cell-independence and will be discussed in further detail in section 13.4.

11.7: Evidence for the presence of another ets-related protein in the ELM system.

Northern blot analysis of the ELM cell lines has demonstrated that the I-1 cell line expresses both the *erg* and the *Fli-1* genes, whilst I-2 only produces *erg* mRNA. These genes encode highly homologous proteins both of which are able to bind to the E74 sequence when they are produced in *in vitro* translation systems and used in EMSAs: nuclear protein extract from I-1 and I-2 cells contain proteins that bind to the E74 oligonucleotide in the same assays. However, without performing 'super-shift' experiments with Fli-1 or erg anti-sera (which are currently unavailable), it is not possible to conclude that the E74 binding activity in the I-1 and I-2 extracts is due to the erg and Fli-1 proteins: other ets-related proteins may be responsible. To examine this problem further, EMSAs were performed using nuclear extract from MS-5 cells, which Northern blot analysis had demonstrated expressed no detectable *erg* mRNA and only an extremely low level of *Fli-1* (Figures 11.4 and 11.7A). Despite presumably containing little, if any, Fli-1 or erg protein, they still contained a complex series of E74-binding activities which exhibited some similarities and some differences from the I-1 and I-2 E74 complexes (Figure 11.11). The bands labelled A and B2 seen in the ELM-I and MS-5 EMSAs comigrated and exhibited very similar competition with the E74, E74(mut), PEA3 and PU-1 oligonucleotides. It seems likely, therefore, that these complexes are formed by the same or highly homologous protein(s) in these extracts, and is strongly indicative that other, possibly unknown,

Figure 11.11: EMSA showing E74 oligonucleotide binding activity in the stromal cell line, MS-5.

Nuclear protein extract was isolated from I-2 and MS-5 cells and ^{32}P-end-labelled double-stranded E74 oligonucleotides prepared (see Methods and Materials). Binding reactions, using 500pg of labelled oligonucleotide and equal quantities of nuclear protein, and the non-denaturing polyacrylamide gel electrophoresis were performed as described in the Methods and Materials. Gels were dried and exposed to X-ray film for 12 hours. The **labelled oligonucleotide** used in all reactions was E74; the unlabelled **competitor** oligonucleotides incorporated into the binding reaction are shown above the autoradiograph: (-) indicates no competitor, (+) indicates 10-fold excess (5ng), (+++) indicates 200-fold excess (100ng).

Low mobility, **non-specific binding**, which is not efficiently competed by unlabelled oligonucleotide, is indicated by the letter '**C**'. Specific complexes are named **A**, **B1**, **B2** and **B3**, and are discussed in the text. **Free oligonucleotide**, which has not been bound by any proteins in the extracts, is seen at the bottom of the autoradiograph.

Oligonucleotide sequences:

PEA3: GATCCTCGAGCAGGAAGTTCGAGATC
(Wasylyk *et al*, 1990).
PU-1: GATCCATAACCTCTGAAAGAGGAACTTGGTTAGGTGATC
(Klemsz *et al*, 1990).
E74: GATCTCTAGCTGAATAACCGGAAGTAACTCATCCTAGGATC
(Reddy and Rao, 1991).
E74(mut):GATCTCTAGCTGAATAACCCAAGTAACTCATCCTAGGATC
(Reddy and Rao, 1991).

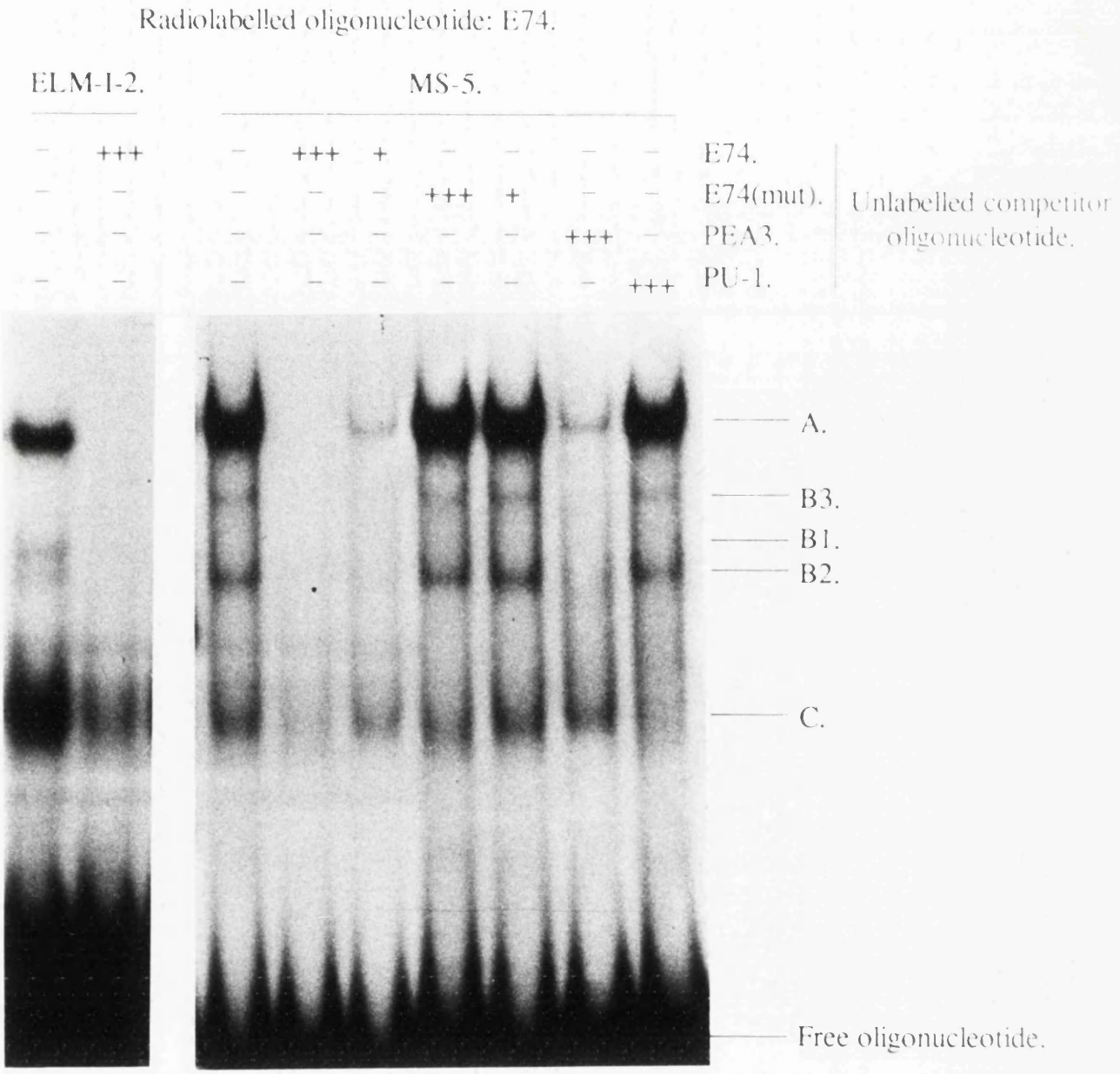

Radiolabelled oligonucleotide: E74.
ELM-I-2.
MS-5.
– +++ – +++ + – – – – E74.
– – – – – +++ + – – E74(mut).
– – – – – – – +++ – PEA3.
– – – – – – – – +++ PU-1.
Unlabelled competitor oligonucleotide.
A.
B3.
B1.
B2.
C.
Free oligonucleotide.

Figure 11.11.

ets-related proteins are present in the I-1 and I-2 extracts along with the Fli-1 and erg proteins. These are not specific for the stromal cell-independent ELM cells since they are also formed in EMSAs using nuclear proteins from D#6 cells growing in contact with MS-5 stromal cells, or freshly-separated from MS-5 cells by centrifugal elutriation (Figure 11.12). The protein(s) in band A appears to be widely expressed in various human and murine cell lines and tissues since a complex with identical mobility and competition profile is observed in murine foetal liver, human K562 myeloid leukaemia cells, C5 cells derived from murine embryonic epithelium, human cervical and liver tumour cell lines and murine STO fibroblasts (J. O'Prey, personal communication).

The B1 band observed with the I-1 and I-2 extracts (Figure 11.2A and B) was not present in the MS-5 EMSAs, although a lower mobility complex, termed B3, was formed (Figure 11.11). Unlike B1, B3 is competed using a large excess of the PEA3 sequence: B1 and B3, therefore, are probably formed by distinct proteins present in each cell type. B1 may be formed by Fli-1 or erg, and coincidentally, this complex does comigrate with E74 bound to *in vitro*-translated Fli-1 protein shown in Figure 11.3. However, B1 is not formed with nuclear extract from D#6 cells grown either with or without MS-5 contact, both of which express the *erg* gene to some extent (Figure 11.12).

In summary, it is possible to draw a number of conclusions from these E74 EMSA results. First, they imply the presence of ets-related protein(s) in the ELM system in addition to Fli-1 and erg, the expression of which appears not to correlate with stromal cell-independent growth. Second, they question the validity of using *in vitro*-translated ets proteins to determine their sequence specificity. Fli-1 and erg both bind E74 in these assays, yet it has proven very difficult to demonstrate binding of these proteins to this sequence in nuclear protein preparations from cell lines expressing the genes. In particular, the inability to detect any differences in E74 binding activity in the *Fli-1*-expressing I-1 cell line compared to the I-2 cells, implies that when *Fli-1* is produced *in vivo*, in a state subject to post-translational

Figure 11.12: EMSA showing E74 oligonucleotide binding activity in the ELM-D#6 cells growing with or without MS-5 contact.

Nuclear protein extract was isolated from a ELM-D#6/MS-5 coculture (ratio of 6 ELM-D#6 cells per MS-5), and also from a pure population of ELM-D#6 cells prepared by centrifugal elutriation (see Methods and Materials). ^{32}P-end-labelled double-stranded E74 oligonucleotides were synthesised as described in the Methods and Materials. Binding reactions, using 500pg of labelled oligonucleotide and equal quantities of nuclear protein, and the non-denaturing polyacrylamide gel electrophoresis were performed as described in the Methods and Materials. Gels were dried and exposed to X-ray film for 12 hours. The **labelled oligonucleotide** used in all reactions was E74; the unlabelled **competitor** oligonucleotides incorporated into the binding reaction are shown above the autoradiograph: (-) indicates no competitor, (+++) indicates 200-fold excess (100ng).

Low mobility, **non-specific binding**, which is not competed by unlabelled oligonucleotide, is indicated by the letter 'C'. Specific complexes are named **A** and **B2**, and are discussed in the text. **Free oligonucleotide**, which has not been bound by any proteins in the extracts, is seen at the bottom of the autoradiograph.

Oligonucleotide sequences:

PEA3: GATCCTCGAGCAGGAAGTTCGAGATC
(Wasylyk *et al*, 1990).
PU-1: GATCCATAACCTCTGAAAGAGGAACTTGGTTAGGTGATC
(Klemsz *et al*, 1990).
E74: GATCTCTAGCTGAATAACCGGAAGTAACTCATCCTAGGATC
(Reddy and Rao, 1991).
E74(mut):GATCTCTAGCTGAATAACCCAAGTAACTCATCCTAGGATC
(Reddy and Rao, 1991).

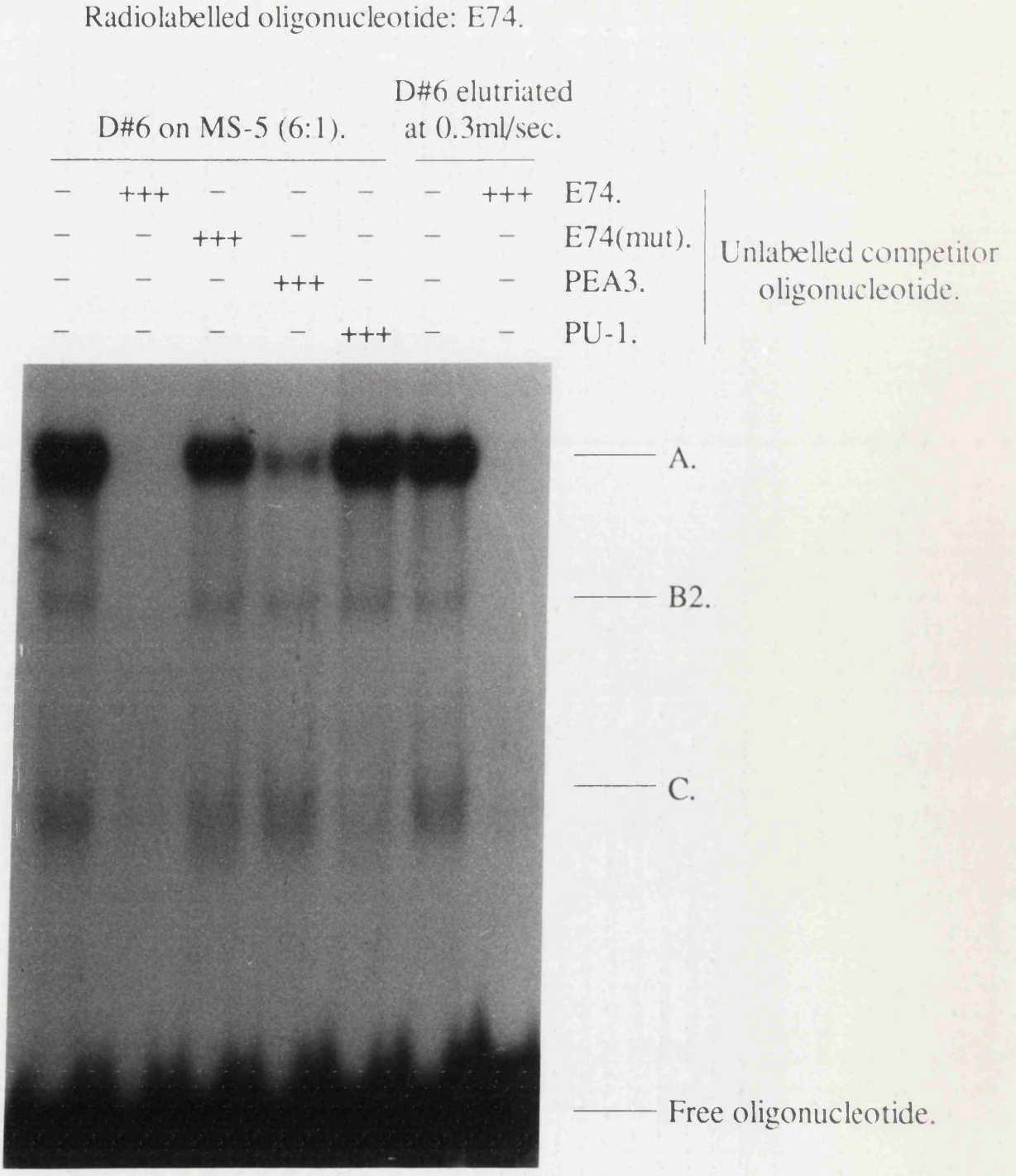

Radiolabelled oligonucleotide: E74.
D#6 on MS-5 (6:1).
D#6 elutriated at 0.3ml/sec.
– +++ – – – – +++ E74.
– – +++ – – – – E74(mut).
– – – +++ – – – PEA3.
– – – – +++ – – PU-1.
Unlabelled competitor oligonucleotide.
A.
B2.
C.
Free oligonucleotide.

Figure 11.12.

modifications and hetero-oligomer formation, the sequence specificity of the protein is altered. There are precedents for this with the elk-1 and SAP-1 proteins that are only able to bind to the recognition sequence in the serum response element after interaction with a SRF dimer (Dalton and Treisman, 1992; Janknecht and Nordheim, 1992; Rao and Reddy, 1992b). However, alternative possibilities are that (a) the *Fli-1* and *erg* mRNAs are not translated in the ELM cell lines, or (b) the Fli-1/E74 and erg/E74 complexes are part of the low mobility band A seen in the EMSAs. To answer these questions, erg and Fli-1 anti-sera must be developed.

CHAPTER 12: Characterisation of a rearrangement at the *Fli-1* locus.

12.1: Southern blot analysis of the *erg* and *Fli-1* loci.

Inappropriate, deregulated expression of a gene in a developing tumour cell can occur either indirectly, as a consequence of alterations in the transcription factor profile of the cell, or directly, by the acquisition of a mutation(s) in the *cis* regulatory sequences of the gene. There are several possible mechanisms whereby a mutational event can increase the expression of a particular gene. For example, transposing a transcriptional enhancer to a position adjacent to the promoter of a gene will alter its normal regulation. In human leukaemias, translocation events have been well characterised and show proto-oncogenes becoming controlled by a cellular enhancer highly active in the cell type the leukaemia is derived from (Sawyers *et al*, 1991). In retrovirally-induced leukaemias studied in animal model systems, insertion of the viral genome into a particular site in the host DNA may confer a selective advantage during tumour progression, since the viral LTR deregulates the expression of a particular gene, either by transcriptional read-through or by enhancer insertion (reviewed in Peters, 1990). These kind of mutations disrupt the gross structure of the DNA and are readily detectable by Southern blot analysis. Smaller, more subtle

mutations in the normal regulatory domains of a gene could possibly have a similar effect on transcription: these are unlikely to be found by such experimentation.

The murine *erg* cDNA probe (426*erg*) generated in section 11.4.1 was used to probe a series of Southern blots of ELM DNA digested with five different restriction endonucleases (*Bam* HI, *ECo* RI, *Kpn* I, *Nco* I and *Xba* I). No structural differences, either amplification or rearrangement, were detected between the normal C3H spleen control and any of the ELM cell lines tested (ELM-D, D#6, I-1, I-2, I-5 and I-6) (data not shown). However, this does not unequivocally exclude the possibility of a mutation directly deregulating *erg* expression in cells of the system in view of the probe being only a partial cDNA derived from the middle of the mRNA.

Similar studies were also performed for *Fli-1*. The *Fli-1* locus has been well characterised by others investigating common retroviral integration sites in MuLV-induced leukaemias (Ben-David *et al*, 1990a, 1991; Bergeron *et al*, 1991). A map of the region, showing the location of these integrations and a number of relevant restriction enzyme cleavage sites, was compiled from these studies (Figure 12.1C). Interestingly, this demonstrated that the orientation of the inserted provirus varies, depending upon which particular MuLV (F-MuLV or Cas-Br-E) was used to induce the leukaemia.. The position of the SU-9 genomic probe obtained from Dr. E. Rassart is also shown. This was used to look for a rearrangement at the *Fli-1* locus in the *Fli-1*-expressing I-1 cells, using C3H spleen as a normal control. Genomic DNA was digested with five restriction enzymes, electrophoresed on a 0.8% agarose gel, blotted onto a nylon membrane and probed with radiolabelled SU-9 (Figure 12.1A). Three of the enzymes (*Pst* I, *ECo* RI and *Bam* HI) produced identical banding patterns in the two DNA samples. However, with *Xba* I and *Hind* III, an additional band is apparent in the I-1 DNA, accompanied by a reduction in the intensity of the normal band. This is consistent with one *Fli-1* allele having undergone a rearrangement, with the breakpoint localised between an *Eco* RI and a *Hind* III site, as shown in Figure 12.1C.

Since the SU-9 probe is approximately 5kb from the breakpoint of the putative rearrangement, an attempt was made to confirm the result using a second probe closer

Figure 12.1: Detection of a rearrangement in one *Fli-1* allele in ELM-I-1 cells by Southern blot analysis.

A and **B**: Genomic DNA was isolated from ELM-I-1 cells (denoted by '**I-1**') and normal C3H spleen tissue (denoted by '**S**') and 20μg aliquots digested with the five restriction endonucleases shown above the autoradiographs. A Southern blot was prepared of these cleaved DNA samples and probed initially with a ^{32}P-labelled SU-9 probe (Bergeron *et al*, 1991) as described in Methods and Materials. A 3 day exposure of this blot is shown in panel **A**; the position of molecular weight DNA markers electrophoresed adjacent to the DNA samples are shown to the right of the autoradiograph. The blot was then stripped and probed with a probe from the non-coding region of the *Fli-1* gene, generated by PCR from spleen genomic DNA (see Materials and Methods). A 6 day exposure of this blot is shown in panel **B**; the position of molecular weight DNA markers electrophoresed adjacent to the DNA samples are shown to the right of the autoradiograph. The background signal on this autoradiograph is particularly high: this is probably a consequence of the short length of the probe (~210bp) used in this experiment. The minor 4kb band seen in the *Bam* HI digest is thought to be a consequence of partial DNA cleavage.

C: Restriction map of the normal *Fli-1* locus for the enzymes used in the Southern blot analysis (derived from Bergeron *et al*, 1991, and Ben-David *et al*, 1990a, 1991). Abbreviations used for the restriction sites are: **P** - *Pst* I; **X** - *Xba* I; **H** - *Hind* III; **E** - *ECo* RI; **B** - *Bam* HI. Also shown as black boxes are the regions where retroviral insertions occur in **F-MuLV** and **Cas-Br-E**-induced leukaemias; the orientation of the integrated viruses is also shown. The empty boxes show the position of the **SU-9** probe (from Bergeron *et al*, 1991), the location of the **non-coding region of the *Fli-1*** gene (as determined from the Southern blot analysis shown in panel B) and the region involved in the rearrangement identifed in the ELM-I-1 cells (labelled '**ELM breakpoint**').

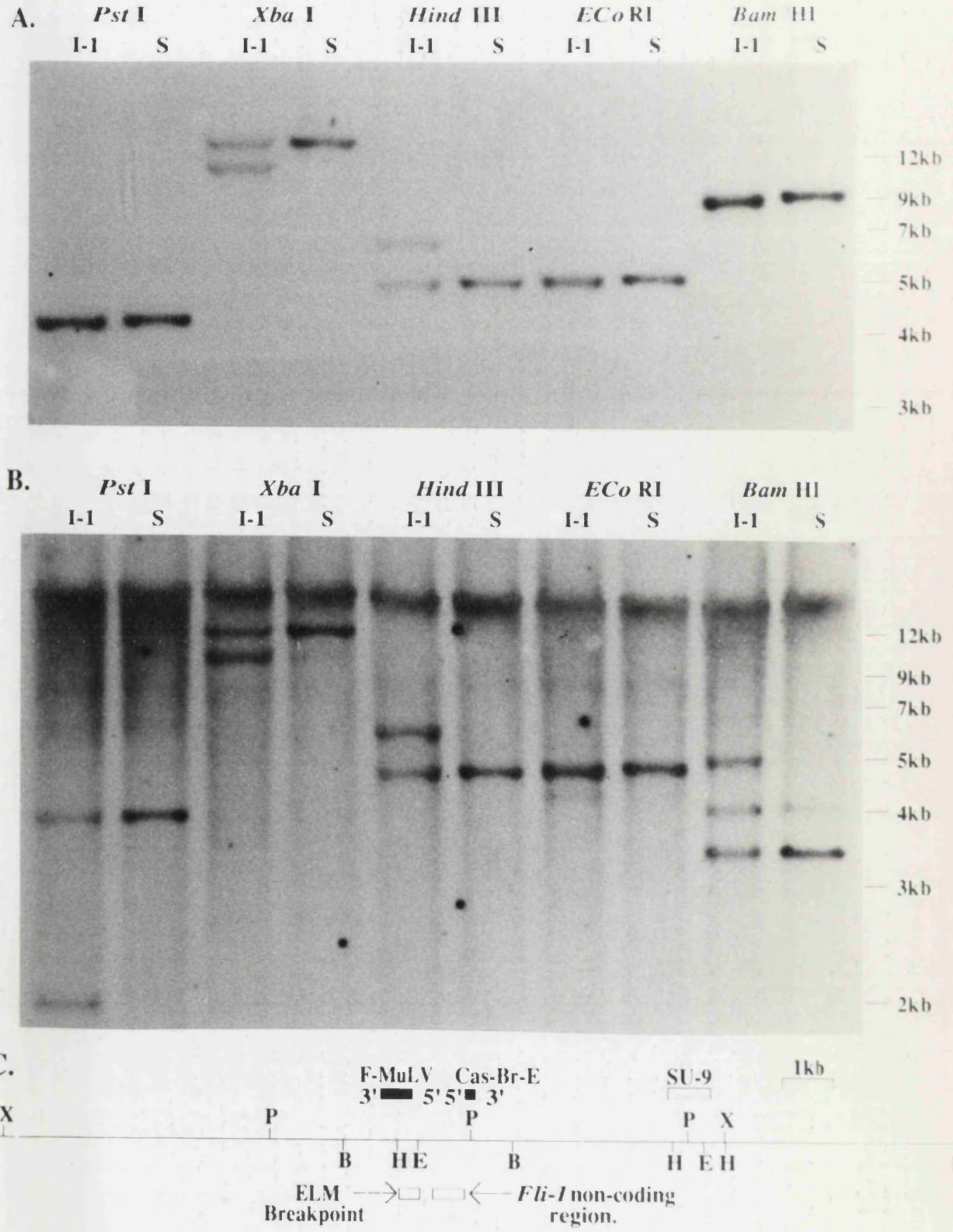

A.
Pst I
Xba I
Hind III
ECo RI
Bam HI
I-1
S
12kb
9kb
7kb
5kb
4kb
3kb
B.
2kb
C.
F-MuLV
Cas-Br-E
3'
5'
SU-9
1kb
X
P
B
H
E
ELM
Breakpoint
Fli-1 non-coding
region.

Figure 12.1.

to this region. Ben-David and his colleagues (1991) have stated that the 5' end of the *Fli-1* cDNA is able to hybridise to a region adjacent to the retroviral insertion sites, between the *ECo* RI and *Bam* HI sites shown in Figure 12.1C. PCR from genomic DNA was therefore used to generate a short 210bp cDNA probe containing the 5' non-coding sequences of *Fli-1*, which was then hybridised to the blot previously probed with SU-9. With all the restriction enzymes shown, except *ECo* RI, I-1 DNA contained an additional band absent from the spleen control (Figure 12.1B). Thus, the 5' non-coding region of *Fli-1* maps very close to the breakpoint found in I-1 DNA, between the *ECo* RI and *Pst* I sites shown on Figure 12.1C. In conclusion, these results demonstrated that a rearrangement had occurred in one *Fli-1* allele in I-1 cells adjacent to the putative first exon of the *Fli-1* gene. The assumed absence of exogenous and endogenous retroviral involvement in the generation and progression of the cells of the ELM system (Itoh *et al*, 1988a), made this rearrangement particularly interesting since it may indicate the presence of a translocation between the *Fli-1* locus and the regulatory regions of another cellular locus.

12.2: Cloning and characterisation of the rearranged *Fli-1* allele.

To elucidate the nature of the rearrangement at *Fli-1*, a genomic library was constructed from I-1 DNA digested with *Sau* 3AI and ligated into λGEM11 (see Methods and Materials). Initially, the library was amplified (section 6.16.4) and $7.5x10^5$ plaques were screened with a radiolabelled SU-9 probe: no positive-hybridising plaques were found, despite the fact that, theoretically, the number of plaques screened had an ~99% probability of containing the required insert (Sambrook *et al*, 1989). The presence of I-1 DNA in the library was confirmed by hybridising to ^{32}P-labelled mouse genomic DNA, and furthermore, three plaques were found that hybridised to the *Fli-1* cDNA fragment excised from the p*Fli* plasmid. However, these were unable to bind to SU-9 (data not shown). These results implied that the breakpoint region was under-represented in the library, an effect that may possibly be the result of amplification. Thus, a fresh library was

constructed and used immediately without amplification. This was screened with SU-9, and two positive clones (A and B) were isolated. The 5' non-coding *Fli-1* cDNA probe was not used in these initial screens because, even though it maps closer to the breakpoint, its short size makes hybridisation more difficult, a point that is illustrated by the intensity of the background signal observed in Figure 12.1B. However, once DNA had been isolated from the two positive clones, this probe was used to show that the clone B insert did not span the breakpoint region. Further analysis of this clone showed that it contained an ~15kb I-1 genomic DNA insert (Figure 12.2A) that was unable to hybridise to any part of the *Fli-1* cDNA, implying the presence of a large first intron (at least 18kb) in the *Fli-1* gene. However, it is possible that alternative-spliced exons not present in the *Fli-1* cDNA cloned in chapter 11 are encoded within this portion.

The cleavage sites for a number of different restriction enzymes were mapped for clone A, using Southern blot analysis and the SU-9 and *Fli-1* 5' non-coding probes, and also by transcription labelling from the SP6 and T7 promoters adjacent to the insert (see Methods, section 6.16.7). Fortunately, this demonstrated that the insert in this 'phage did span the breakpoint and was derived from the rearranged allele. Of the 12.5kb insert in clone A, 10.5kb had a restriction map identical to the wild-type *Fli-1* allele (Figure 12.2A). Three restriction fragments around the breakpoint were excised from the clone and subcloned into the pBluescriptII KS+ plasmid to create the pCB10, pCB20 and pCB30 vectors (Figure 12.2A). These inserts were partially sequenced using primers which hybridise to a region in the pBluescriptII KS+ plasmid adjacent to the cloning site. This showed that the whole 5' non-coding region of the gene and the first six codons, which presumably comprise the first exon of the gene, are found approximately 900bp from the breakpoint, and are transcribed away from this alteration (Figure 12.2B). Interestingly, the viral integrations described by Bergeron *et al* (1991) are all in this first exon, and in the same transcriptional orientation as the gene. Thus, they probably activate *Fli-1* expression by a transcriptional read-through mechanism: this has recently been confirmed (Bergeron

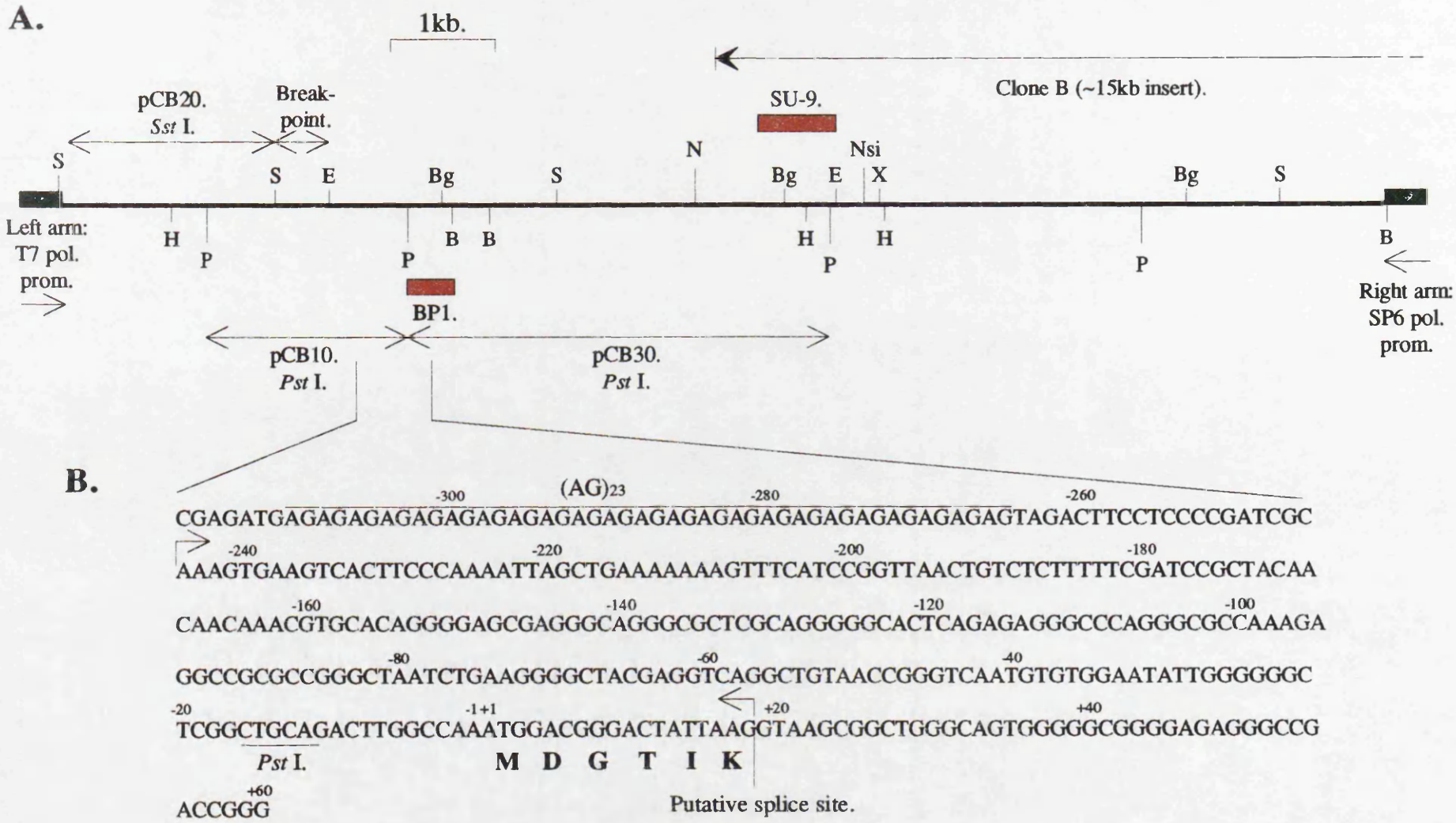
Figure 12.2: Characterisation of a 'phage clone spanning the breakpoint at the *Fli-1* locus.

A library was prepared from the genomic DNA of the ELM-I-1 cells and the λGEM11 bacteriophage vector, and screened with the SU-9 probe (Methods and Materials). Two positive hybridising clones (A and B) were isolated and DNA extracted.

A: Restriction map of clone A. Using a combination of SU-9, the non-coding region probe from the *Fli-1* gene and probes prepared by transcription labelling from the **SP6** and **T7** promoters in the 'phage arms, restriction maps for a number of enzymes were prepared for the two clones (Methods and Materials). Preliminary analysis determined that clone B did not span the breakpoint and was not subjected to any further analysis: its position is shown above the map for clone A. Abbreviations used for the restriction sites are: **P** - *Pst* I; **X** - *Xba* I; **H** - *Hind* III; **E** - *ECo* RI; **B** - *Bam* HI; **S** - *Sst* I; **Bg** - *Bgl* II; **N** - *Nco* I; **Nsi** - *Nsi* I. The positions of the **SU-9** probe (Bergeron *et al*, 1991), and the **BP1** probe used in later experiments, are denoted by red boxes; the **right and left arms** of the 'phage vector are given as green boxes. The position of the **breakpoint** is labelled accordingly. The three DNA fragments which were subcloned into pBluescriptII KS+ for further sequence analysis (Methods and Materials) are shown as double-headed arrows, adjacent to which is the name of the vector formed (**pCB10**, **pCB20** or **pCB30**) and the restriction enzyme used to cleave the fragment from the 'phage clone.

B: Sequence of the putative first exon of the *Fli-1* gene, as derived from the pCB10 and pCB30 vectors. Sequence reactions and denaturing polyacrylamide gel electrophoresis are described in the Methods and Materials. The region between the two **arrows** had already been published (Ben-David *et al*, 1991): no nucleotide differences were apparent between the sequence from this reference and the one shown here. The **first six codons** are indicated by the bold letter beneath them which represent the amino acid for which they code: the **putative splice site** is shown. The ***Pst* I** restriction site in this region is underlined. Nucleic acid numbering takes the adenine residue of the initiating ATG as **+1**. The dinucleotide repeat is labelled with **(AG)$_{23}$**.

Figure 12.2.


A.
1kb.
Clone B (~15kb insert).
pCB20.
Sst I.
Break-
point.
SU-9.
S
S
E
Bg
S
N
Nsi
Bg
E
X
Bg
S
Left arm:
T7 pol.
prom.
H
P
P
B
B
H
P
H
P
B
Right arm:
SP6 pol.
prom.
BP1.
pCB10.
Pst I.
pCB30.
Pst I.
B.
(AG)23
-300
-280
-260
CGAGATGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGTAGACTTCCTCCCCGATCGC
-240
-220
-200
-180
AAAGTGAAGTCACTTCCCAAAATTAGCTGAAAAAAAGTTTCATCCGGTTAACTGTCTCTTTTTCGATCCGCTACAA
-160
-140
-120
-100
CAACAAACGTGCACAGGGGAGCGAGGGCAGGGCGCTCGCAGGGGGCACTCAGAGAGGGCCCAGGGCGCCAAAGA
-80
-60
-40
GGCCGCGCCGGGCTAATCTGAAGGGGCTACGAGGTCAGGCTGTAACCGGGTCAATGTGTGGAATATTGGGGGGC
-20
-1+1
+20
+40
TCGGCTGCAGACTTGGCCAAATGGACGGGACTATTAAGGTAAGCGGCTGGGCAGTGGGGGCGGGGAGAGGGCCG
Pst I.
M D G T I K
+60
ACCGGG
Putative splice site.

et al, 1992). However, the F-MuLV integrations discovered by Ben-David and his colleagues (1990a) are further upstream outside the exon and in the opposite orientation. In this case, the gene presumably becomes over-expressed by an enhancer insertion mechanism. Twenty bases upstream from the beginning of the published sequence for *Fli-1* was found a region consisting of 23 repeats of the dinucleotide, AG. The function of dinucleotide repeats of this kind is unclear, although *in vitro* DNA fragments consisting solely of these sequences are able to form left-handed double helical structures, termed Z-DNA (reviewed in Hill, 1991). Whether this occurs *in vivo* remains an issue of some controversy, but it has been proposed that these structures are involved in recombination and transcriptional control. The position of the AG repeat in the *Fli-1* locus adjacent to the first exon of the gene, implies that it may play a role in regulating *Fli-1* gene expression.

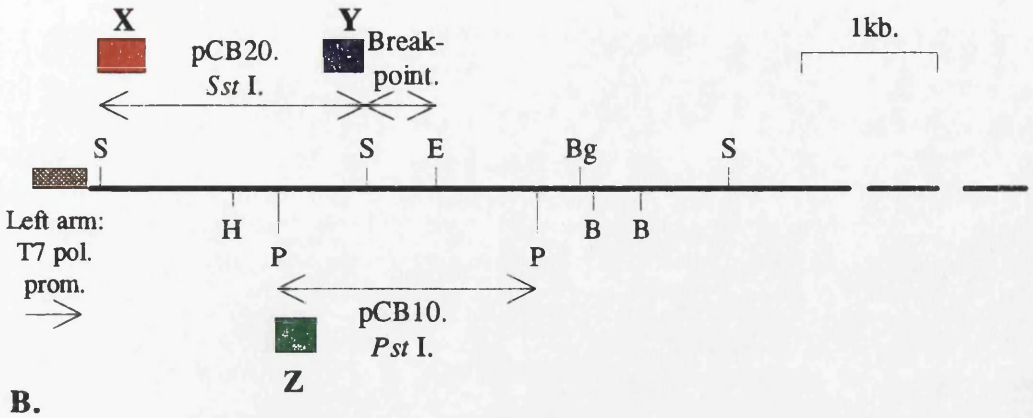
Sequence from the 2kb portion of clone A from the other side of the breakpoint, derived from the regions X, Y and Z of the pCB10 and pCB20 plasmids (Figure 12.3A), was compared with known sequences in the GenBank data-base, using GCG sequence analysis software (Devereux *et al*, 1984). The best match, of nearly 100%, was with the LTR and *gag* gene of the exogenous F-MuLV retrovirus strain FB29 (Figure 12.3B), the sequence of which had been published by Perryman *et al* (1991). It remained a possibility, however, that the provirus inserted at the *Fli-1* locus is derived from endogenous retroviral sequences, since in a number of radiation-induced leukaemias endogenous viruses become active, and are involved in deregulating genes necessary for tumour progression (Janowski and Boniver, 1986). To test for the presence of exogenous F-MuLV insertions in the genomic DNA of I-1 cells, a DNA probe specific for the *env* gene of the virus (termed *ENV*; see Table 6.1 in the Methods and Materials) was hybridised to a Southern blot of *Bgl* II-cleaved DNA from these cells, using C3H mouse spleen DNA as a control. The combination of the *ENV* probe and the *Bgl* II digest will create junction fragments between the virus and the host DNA, allowing the number of proviruses to be calculated. No hybridisation to spleen DNA was observed, as expected, but three bands were

Figure 12.3: Comparative analysis of sequence derived from the rearranged *Fli-1* allele isolated from ELM-I-1 cells, and the retrovirus F-MuLV.

A: Restriction map of the left-hand end of clone A depicted in Figure 12.2. Abbreviations used for the restriction sites are: **P** - *Pst* I; **H** - *Hind* III; **E** - *ECo* RI; **B** - *Bam* HI; **S** - *Sst* I; **Bg** - *Bgl* II. The **left arm** of the 'phage vector is given as a stippled box. The position of the **breakpoint** is labelled accordingly. Two DNA fragments which were subcloned into pBluescript for further sequence analysis (Methods and Materials) are shown as double-headed arrows, adjacent to which is the name of the vector formed (**pCB10** or **pCB20**) and the restriction enzyme used to cleave the fragment from the 'phage clone. The three boxes labelled **X**, **Y** and **Z** are the regions of these two vectors which were sequenced.

B: Sequence from regions **X**, **Y** and **Z** compared to F-MuLV (strain FB29) (Perryman *et al*, 1991). The sequence from the 'phage subclones is the top line in each case with F-MuLV below. A dash (-) indicates nucleic acid identity, an asterisk (*) shows where a base is missing in the F-MuLV sequence, and bases missing from the clone A sequence but present in F-MuLV are given below both sequences with a line showing where the difference lies.

A.



B.

X

```
CTTCCCTCACTAAGTCTCCTAAGGTCCTGCTCTTCAAATCTTCTAACCGCTCTAACTTTCG
----------------------------T--------T-------------------------

CCCGATATCCGGGGCGGACTGCCAGATGAATGACATGGCCACATTGGTTTCTTGCCCTGGG
------------------------------------------------------------

TCCTCAGGGTCATAAGGAGTGTATCTGCGATAGGCCTCCTTGAGTCTCTCTAAAAAGGCTG
------------------------------------------------------------

AGGGAGACTCATTAGGTCCCTGGGTTATCCCTTTTACCTTGGCCAAATTGGTGGG
--------------------------------------------------------
```

Compared to the *gag* gene of F-MuLV (strain FB29).

Y

```
CCTGTGGTCGTAGTACCTGGCAGGTCTCAGATCCGGACGAGCCCAAATGAAAGACCCCCGAG
-----------------------|---------|---------|--------------------
                       AG        C         CC

ACGGGTAGTCAATCACTCTGAGGAGACCCTCCCAAGGAACAGCGAGACCACAAGTCGGATGC
-----------------------------------------------------G----------

AACAGCAAGAGGCTTTATTGGATACACGGTACCCGGGCGACACAGTCTATCGGAGGACTGGC
----------------------*G--------------T--------------------
                       |
                       GA

GCGCCGAGTGAGGGGTTGTGGGCTCTTTTATAGAGCTC
-------------------A----------------
```

Compared to the F-MuLV (strain FB29) LTR.

Z

```
GCTTCGGCTCCAAACCGAAAGCAAAAATTTAGACGGGGGCGGAAACCGTTTCAGCCCTCCG
-------T----------------------C------A----------T----T----T----

TCCCCTACCAGAACCACATACCCCTCCTCTAAAGGGTGTGCACCAAAGAGTCCAAAACGAT
--------------------------------G---G----------------------G-

CGGGATATTTGGACTCTGGCCGGGCCACAAAAATGGCCCCCGAAGTCCCTGGGACGTCTCC
------GG-----------------------------------------T-----------

CAGGGTTGCGGCCGGGTATCCGAACTCGTCAGTTCTACCACGGGTCCGCCAGATACAG
-----------------G--*----C---------C----------------G-----
```

Compared to the 5' end of the *gag* gene, and part of the LTR, of F-MuLV (strain FB29).

Figure 12.3.

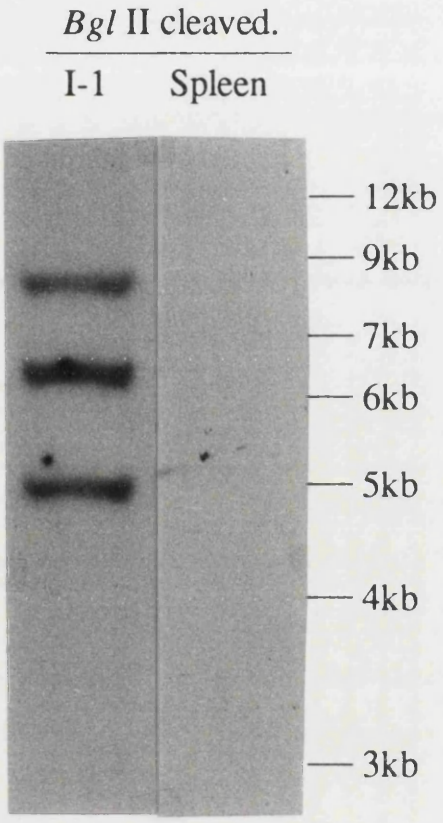
detected in the I-1 DNA, implying that three F-MuLV proviruses were present in this cell line (Figure 12.4). This result confirmed that exogenous virus was present in these cells. It appears, therefore, that *Fli-1* became expressed in the ELM-I-1 cell line as a consequence of the insertion of F-MuLV. Since the viral genome is in the opposite transcriptional orientation to *Fli-1*, the LTR presumably acts as an enhancer for the normal *Fli-1* promoter.

12.3: Retroviral expression and integration in the ELM system.

The presence of F-MuLV in I-1 was surprising since the history of the development of the original ELM tumour and the cell lines within the system contains no mention of a viral involvement (Itoh *et al*, 1988a, 1988b, 1993). Thus, the RNA and DNA of the other cells in the system was tested, to examine when infection occurred. When RNA was hybridised to the *ENV* probe by Northern blot analysis, it was obvious that all the ELM-D and ELM-I cell lines, and indeed the *in vivo* passaged tumour cells, expressed variable amounts of viral gene transcripts (predominantly 10kb and 4kb species) that were absent from the normal C3H mouse tissue controls (Figure 12.5). The nature of the 9kb transcript restricted to the D#6, I-5 and I-6 samples, is unknown, but may represent the presence of recombinant retroviruses formed between exogenous and endogenous retroviral sequences. Not only are viral transcripts produced, but it is also possible to observe virus budding from ELM cells under the electron microscope (Ostertag, personal communication). Exactly how virus infection occurred is not known, but the viral gene expression in the *in vivo* passaged tumour cells suggests that it was involved in generating the ELM tumour, in a similar way to erythroleukaemias previously described by Ben-David and his co-workers (1990a, 1991). This implied that the ELM leukaemia was not induced solely by X-ray irradiation, as originally thought (Itoh *et al*, 1988a), but suggested that F-MuLV infection was probably also involved in leukaemic development and phenotypic change within the cell lines. However, SFFV, the other component of the Friend virus complex, was not present in the ELM system. This was shown by

Figure 12.4: Southern blot demonstrating the presence of exogenous F-MuLV-like retroviruses in the ELM-I-1 cell line.

Genomic DNA was isolated from ELM-I-1 cells (denoted by '**I-1**') and normal C3H spleen tissue ('**spleen**') and 20μg aliquots digested with *Bgl* II. A Southern blot was prepared of these cleaved DNA samples and probed with a ^{32}P-labelled *ENV* probe derived from the *envelope* gene of F-MuLV as described in Methods and Materials. A 3 day exposure of the blot is shown; the position of molecular weight DNA markers electrophoresed adjacent to the DNA samples are shown to the right of the autoradiograph.



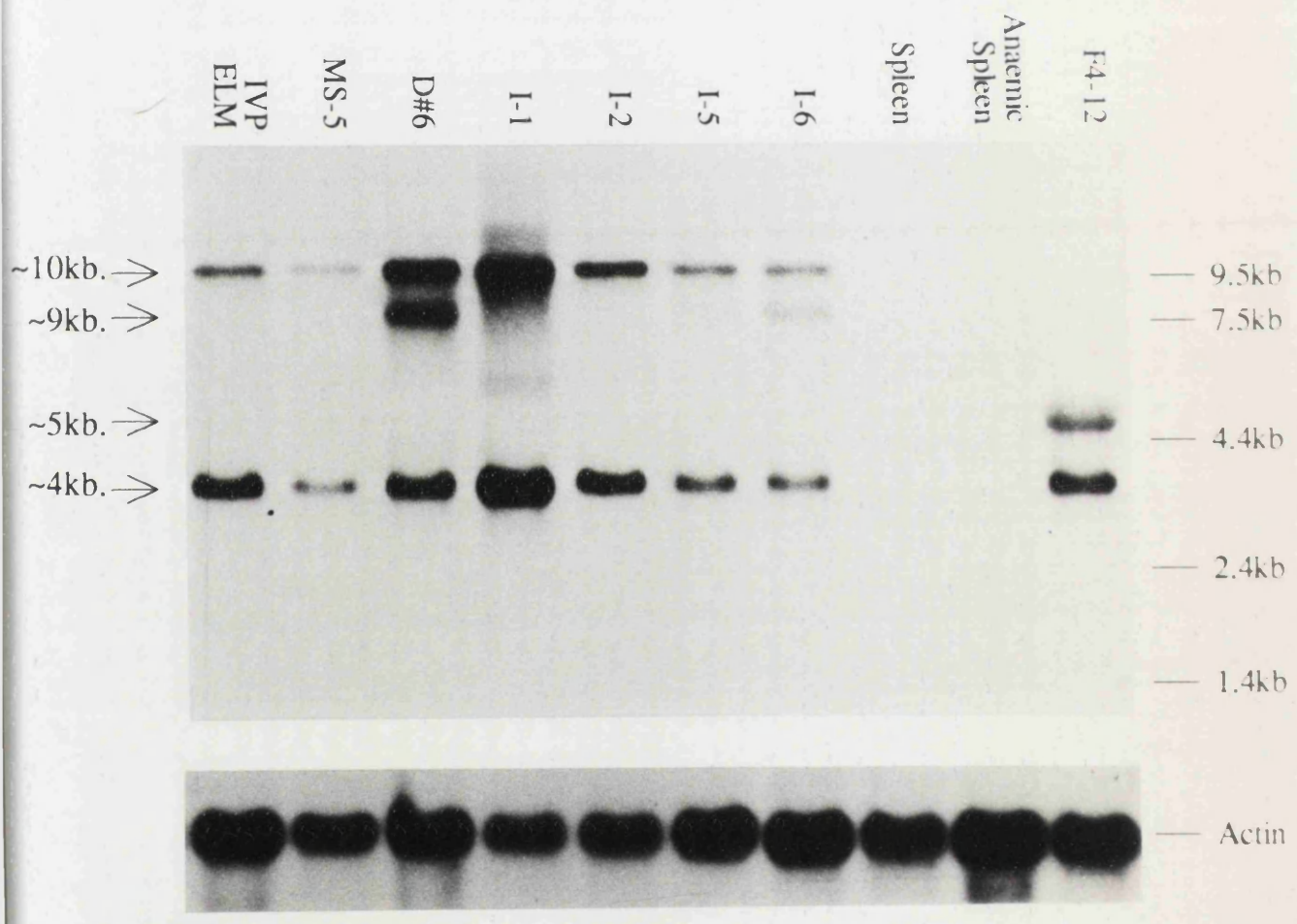
Probe: F-MuLV *env* gene.

Figure 12.4.

Figure 12.5: Expression of mRNAs hybridising to the F-MuLV *envelope* gene in the ELM system.

20μg of total cellular RNA was electrophoresed, blotted and hybridised to a ^{32}P-labelled probe derived from the ***envelope*** gene of F-MuLV (Methods and Materials). Exposure time: 12 hours. Molecular weight markers are given, in kb, to the right of the autoradiograph. The position of the four different transcripts which hybridise to this probe are indicated by the arrows to the left of the autoradiograph, with an approximation of their size. The blot was stripped and reprobed with an *actin* cDNA to assess the relative levels of RNA loading. Exposure time: 4 hours.

Tissues and cell lines examined were:
I-1, I-2, I-5 and **I-6**:- Stromal cell-independent clones I-1, I-2, I-5 and I-6.
D#6:- ELM-D#6 cells growing on live MS-5 cells (ratio 6 ELM-D#6 cells per MS-5).
MS-5:- Stromal cell line, MS-5.
IVP ELM:- *In vivo* passaged ELM tumour cells.
Spleen:- Normal C3H mouse spleen.
Anaemic spleen:- Erythropoietically-active spleen from a C3H mouse which had been treated with phenylhydrazine to induce anaemia (see Methods and Materials).
F4-12:- Friend virus-induced erythroleukaemia cell line, F4-12.



Probe: F-MuLV *env* gene.

Figure 12.5.

Northern and Southern blot analysis using a DNA probe from the *gp55* gene of the virus (see Table 6.1 in the Methods section). Although the probe hybridised to endogenous retroviral sequences, compared to normal C3H spleen there was neither any detectable novel integrations nor any significant increase in the expression of the *gp55* gene in the *in vivo* passaged ELM cells or in the cell lines D#6, I-1, I-2, and I-6: F4-12 DNA, used as a positive control, contained a number of integrated SFFV proviruses and exhibited a large over-expression of the *gp55* gene. However, as will be described below, the I-5 cell line may contain recombinant viruses formed with endogenous *gp55*-like sequences.

Surprisingly, further Southern blot analysis with the F-MuLV *ENV* probe showed that the number of insertions varied between the different ELM cells: two insertions occur in the *in vivo* passaged tumour cells, but this increases up to nine in the cell lines derived from them (Figure 12.6). The two stromal cell-dependent cell lines tested had seven proviruses each; I-1 only three; I-2 and I-5 had eight each and I-6 had nine. The additional integrations possibly arise as a consequence of re-infection of the cells with F-MuLV produced either by the leukaemic cells, or by the MS-5 stromal cells which also surprisingly express low amounts of F-MuLV mRNAs (Figure 12.5). (The origin of MS-5 viral infection is also unknown: these cells were reportedly generated by irradiating long-term bone marrow cultures (Itoh *et al*, 1989)). Furthermore, since the restriction endonucleases used will cleave the DNA to create junction fragments between the provirus and the host, it is apparent that only one site remains constant throughout the whole system (labelled 'common band' in Figure 12.6), although I-5 and I-6 contained all the same bands as the D#6 cell line from which they were derived (see also Figure 5.1 in the introduction, chapter 5). Thus, the additional insertion sites in I-5 and I-6 may be involved in generating stromal cell-independent growth.

Another interesting observation is that the I-1 cells contain only one integration that is present in the cells from which they were derived (ELM-D and *in vivo* passaged tumour cells) (Figures 12.6 and 12.9B). Furthermore, it would appear

Figure 12.6: Retroviral integrations in the ELM system detected by Southern blot analysis of DNA cleaved with restriction enzymes which create junction fragments.

Genomic DNA was isolated from the ELM cells, cleaved with either *Bgl* II (panel **A**) or *Nco* I (panel **B**) and Southern blots prepared according to Methods and Materials. Both blots were hybridised to a probe derived from the ***envelope*** gene of the F-MuLV retrovirus and exposed to X-ray film for four days. The position of molecular weight DNA markers electrophoresed adjacent to the DNA samples are shown to the right of the autoradiographs. The '**common band**', which is present in all the samples, is labelled with an arrow shown to the left of the autoradiograph. Spleen DNA from a normal C3H mouse contains no sequences which hybridise to the F-MuLV *envelope* probe (Figure 12.4) was omitted from the blot.

Tissues and cell lines examined were:
IVP ELM:- *In vivo* passaged ELM tumour cells.
D:- ELM-D cells growing on live MS-5 cells (ratio 6 ELM-D#6 cells per MS-5).
D#6:- ELM-D#6 cells growing on live MS-5 cells (ratio 4 ELM-D#6 cells per MS-5).
I-1, **I-2**, **I-5** and **I-6**:- Stromal cell-independent clones I-1, I-2, I-5 and I-6.

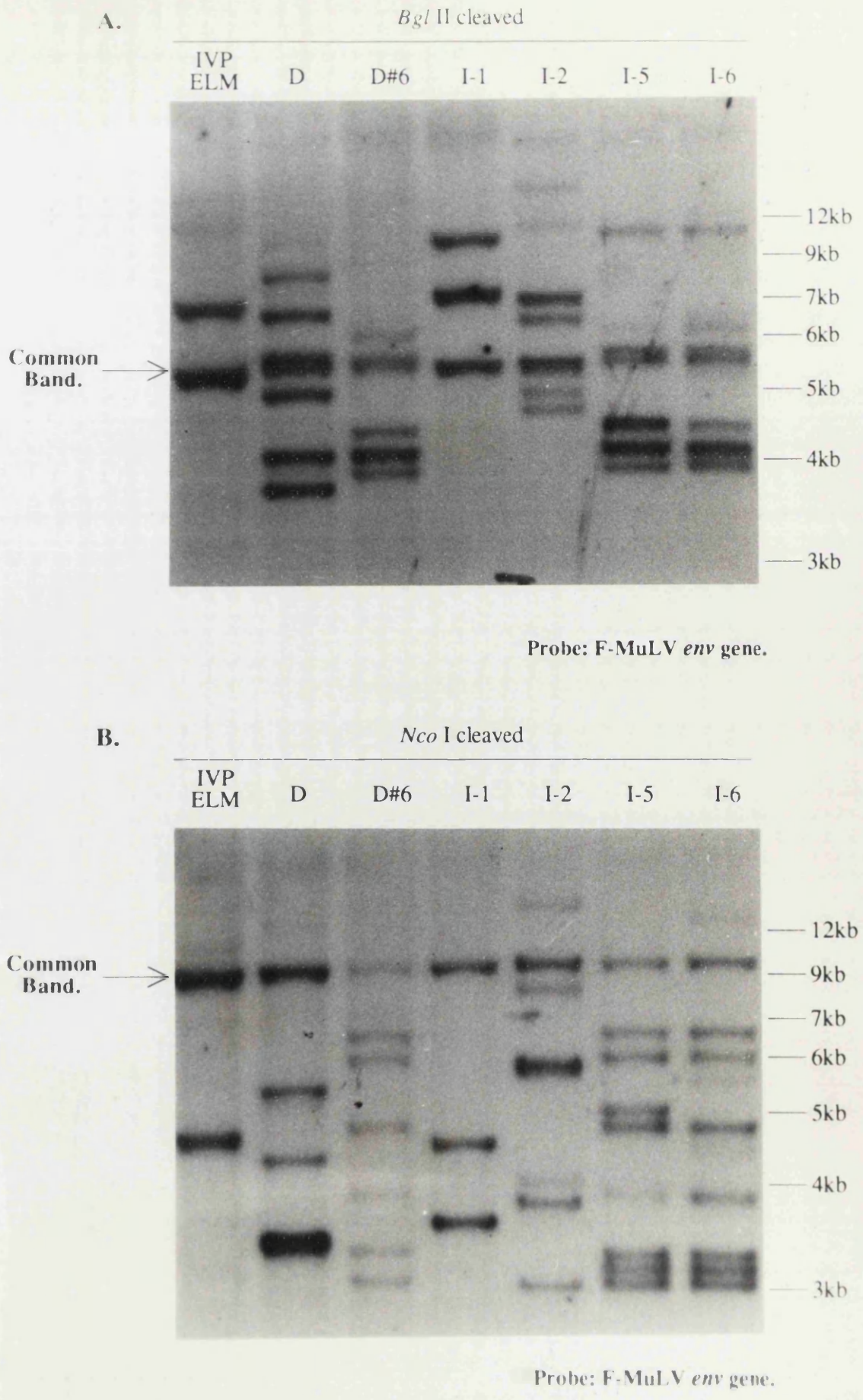

A.
Bgl II cleaved
IVP ELM
D
D#6
I-1
I-2
I-5
I-6
12kb
9kb
7kb
6kb
5kb
4kb
3kb
Common Band.
Probe: F-MuLV env gene.
B.
Nco I cleaved
IVP ELM
D
D#6
I-1
I-2
I-5
I-6
12kb
9kb
7kb
6kb
5kb
4kb
3kb
Common Band.
Probe: F-MuLV env gene.

Figure 12.6.

that I-1 has actually lost some of the inserted proviruses in ELM-D cells. Alternatively, I-1 may have been derived from ELM-D at a time when it too only had three insertions, but that continued passage on F-MuLV-infected MS-5 cells caused a gradual re-infection. However, it is difficult to explain why these integrations appear to be present in all ELM-D cells, unless one assumes that they confer a growth advantage. A further contributory factor that may explain these differences is genomic rearrangement, or deletion, of the provirus. In this way, the position of bands observed in one particular cell line could be shifted, or lost, in another. This would not be surprising since tumour cells often have particularly unstable genomes, a property which may, in part, be due to mutation of the *p53* gene (Kastan *et al*, 1992; Yin *et al*, 1992; Livingstone *et al*, 1992). Recombination between exogenous retroviruses and endogenous retroviral sequences occurs in many malignancies induced by viral infection and may be important in the progression of the tumour due to the production of more leukaemogenic viral subtypes. For example, in F-MuLV-induced neoplasms, Friend mink cell focus-forming viruses (F-MCF) are formed which are thought to produce envelope proteins which are able to activate the erythropoietin receptor in a fashion similar to the gp55 protein of SFFV (Ruscetti *et al*, 1981; Chesebro *et al*, 1983, 1984; Li and Baltimore, 1991). In order to examine the ELM system for similar recombination events, two DNA probes were obtained from Ostertag and his colleagues. The first, termed F-MCF *ENV*, is able to hybridise to a wide variety of endogenous retroviral sequences, including those involved in forming Friend-MCF virus in F-MuLV-infected cells (Table 6.1 in Methods and Materials; Oliff *et al*, 1983). No extra bands were observed in the ELM system, that were distinct from those found in C3H spleen DNA, when Southern blots of DNA digested with either *Bgl* II or *Xba* I were hybridised to the probe (data not shown). However, the second probe, a 600bp fragment derived from the *gp55* gene of SFFVp, binds to numerous fragments present in *Bgl* II-cleaved C3H spleen DNA, but interestingly an additional band is apparent in I-5 DNA cleaved with the same enzyme that is absent from the other ELM cell lines tested (Figure 12.7A). A similar

Figure 12.7: Southern and Northern blot analysis of the ELM system using an endogenous retroviral probe derived from SFFV.

A: Genomic DNA was isolated, cleaved with *Bgl* II and a Southern blot prepared according to Methods and Materials. This was hybridised to a probe derived from the ***gp55 envelope*** gene of the SFFV retrovirus (see table 6.1 in the Methods and Materials) and exposed to X-ray film for four days. The position of molecular weight DNA markers electrophoresed adjacent to the DNA samples is shown to the right of the autoradiograph. The **I-5-specific fragment** is labelled with an arrow to the right of the autoradiograph at ~2.9kb. Novel integration sites of SFFV are apparent in the SFFV-induced erythroleukaemia cells, F4-12.

B: 20μg of total cellular RNA was electrophoresed, blotted and hybridised to a ^{32}P-labelled probe derived from the ***gp55 envelope*** gene of $SFFV_p$ (Methods and Materials). Exposure time: 72 hours. Molecular weight markers are given, in kb, to the right of the autoradiograph. The transcript observed in all the cells examined, including normal C3H spleen, is labelled with the letter '**A**' and an arrow. The additional transcript seen in I-5 RNA is labelled with the letter '**B**' and an arrow. The blot was stripped and reprobed with an *actin* cDNA to assess the relative levels of RNA loading. Exposure time: 4 hours.

Cell lines and tissues tested were:
I-1, I-2, I-5 and **I-6**:- Stromal cell-independent clones I-1, I-2, I-5 and I-6.
MS-5:- Stromal cell line, MS-5.
D#6:- ELM-D#6 cells growing on live MS-5 cells (ratio 6 ELM-D#6 cells per MS-5).
IVP ELM:- *In vivo* passaged ELM tumour cells (late passage).
Spleen RNA from a C3H mouse.
F4-12:- Friend virus-induced erythroleukaemia cell line, F4-12.

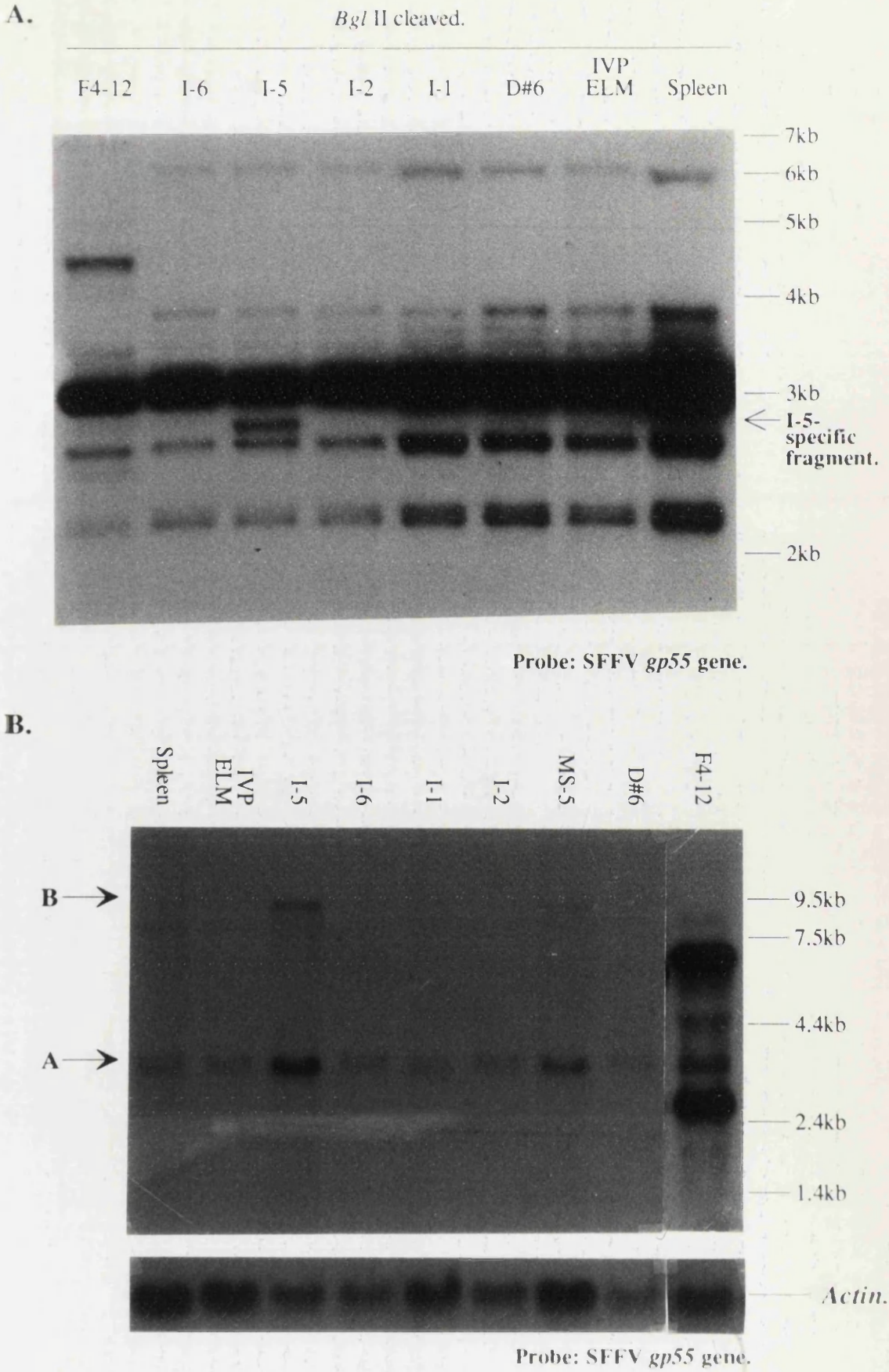

A.
Bgl II cleaved.
F4-12
I-6
I-5
I-2
I-1
D#6
IVP ELM
Spleen
7kb
6kb
5kb
4kb
3kb
I-5-specific fragment.
2kb
Probe: SFFV gp55 gene.
B.
Spleen
IVP ELM
I-5
I-6
I-1
I-2
MS-5
D#6
F4-12
B
A
9.5kb
7.5kb
4.4kb
2.4kb
1.4kb
Actin.
Probe: SFFV gp55 gene.

Figure 12.7.

result is seen when the DNA is cleaved with *Nsi* I or *Nco* I (data not shown). F4-12 cells also had an additional band but in a different position (~4.5kb), presumably due to insertion of the SFFV retrovirus that was used to generate this cell line.

The significance of the I-5-specific band is unclear. It seems highly unlikely that this cell line alone became infected with SFFV, and a more plausible explanation is that it arose as a result of a recombination event between endogenous *gp55*-like sequences and, possibly, the exogenous F-MuLV. Interestingly, a Northern blot hybridised to the same probe showed that I-5 produced significantly more of the ~4kb *gp55*-like mRNA than the other ELM cell lines and normal spleen tissue, although it was considerably less than the SFFV-infected F4-12 cells (Figure 12.7B). I-5 cells also produce a larger transcript of approximately ~10kb that is absent from all the other ELM cell lines tested.

The I-5 cell line is unique in the ELM system, in that it is still able to differentiate in response to epo, but is highly tumorigenic. Whether this is due to some extent to the alterations in the expression of *gp55*-like transcripts is not known: it is possible that the production of MCF-like viruses could enhance the splenomegaly induced by inoculation of I-5 cells by infecting and stimulating the proliferation of host erythroid cells. This could be examined by testing whether the cells in the enlarged spleens all contain the 18bp deletion in the *p53* gene characteristic of ELM cells.

12.4: A full-length F-MuLV provirus is inserted into the *Fli-1* locus in all ELM cells.

The discovery of inserted F-MuLV proviruses in the cells of the ELM system raises a number of important questions concerning the generation of the ELM leukaemia, and its progression to various phenotypically-distinct cell types *in vitro*. The clonality of the *in vivo* passaged tumour cells with respect to its two integration sites, implies that these are important for the tumorigenicity of these cells *in vivo*, and were probably involved in its generation. Furthermore, the conservation of the

integrity of one of these integrations throughout the ELM system suggests that this particular insertion is necessary to maintain the viability of the ELM cells *in vitro*. Obviously, identifying the position of this insertion and determining which gene(s) are affected by the provirus was of interest to further understand the development and progression of murine erythroleukaemias.

One possibility was that F-MuLV insertion into the *Fli-1* locus was an early event in the ELM system that was maintained throughout the various cell lines. This seemed unlikely since the *Fli-1* gene was only expressed in the I-1 cell line. Surprisingly, however, by using radiolabelled BP1 probe isolated from clone A (see Figure 12.2A), it was demonstrated by Southern blot analysis that the same genomic rearrangement is detectable at the *Fli-1* locus in all the ELM cells (Figure 12.8 and 12.9A). Despite the restriction of *Fli-1* expression to the I-1 cell line, no gross structural differences were apparent between this and the other cell lines with the three restriction endonucleases used, *Bgl* II, *Nco* I and *Nsi* I. Since *Nsi* I, does not cleave within the published sequence of F-MuLV (Perryman *et al*, 1991), the fact that it produced a rearranged band approximately 9-9.5kb larger than the wild-type band (Figure 12.9A) suggests that a full-length F-MuLV provirus (~9.25kb) is present at the *Fli-1* locus in all ELM cells. When this blot is stripped of the BP1 probe, and hybridised with radiolabelled F-MuLV *ENV* (Figure 12.9B), the common ELM insertion site described above, was found to comigrate with the rearranged band detected with BP1 (Figure 12.9A). This suggested that the invariant insertion is the one at *Fli-1*, a conclusion that is supported by the *Bgl* II and *Nco* I blots probed with *ENV* (Figure 12.6). This is illustrated in Figure 12.10 which shows a hypothetical map of a full-length F-MuLV provirus in the *Fli-1* locus with the relevant restriction sites marked. The *ENV* probe should theoretically hybridise to a 9kb *Nco* I junction fragment and 5.8kb fragment upon cleavage with *Bgl* II. These are the sizes of the invariant band seen in the ELM system on the *Nco* I and *Bgl* II blots (Figure 12.6).

Thus, in summary, it appears that a full-length F-MuLV provirus is inserted into the *Fli-1* locus in ELM tumour cells that have been passaged *in vivo* and in all the

Figure 12.8: Southern blot analysis demonstrating that the retrovirus inserted in the *Fli-1* locus is present in all the cells of the ELM system.

The two blots previously hybridised to the F-MuLV *envelope* probe (Figure 12.6) were stripped and probed with the BP1 fragment derived from 'phage clone A (Figure 12.2). This probe maps very close to the integration site identified in one *Fli-1* allele in ELM-I-1 cells. Blots were exposed for four days. The position of the '**normal allele**' bands was determined from previous Southern blots of *Bgl* II- and *Nco* I-cleaved C3H spleen genomic DNA which are not shown here: the '**rearranged allele**' bands are also labelled. The differences in the intensity of the bands between lanes is due to variation in DNA loading: the differences apparent within a single lane (**D** and **D#6**) are due to the stromal cell contamination in these samples. The position of molecular weight DNA markers electrophoresed adjacent to the DNA samples are shown to the right of the autoradiographs.

Tissues and cell lines examined were:
IVP ELM:- *In vivo* passaged ELM tumour cells.
D:- ELM-D cells growing on live MS-5 cells (ratio 6 ELM-D#6 cells per MS-5).
D#6:- ELM-D#6 cells growing on live MS-5 cells (ratio 4 ELM-D#6 cells per MS-5).
I-1, **I-2**, **I-5** and **I-6**:- Stromal cell-independent clones I-1, I-2, I-5 and I-6.

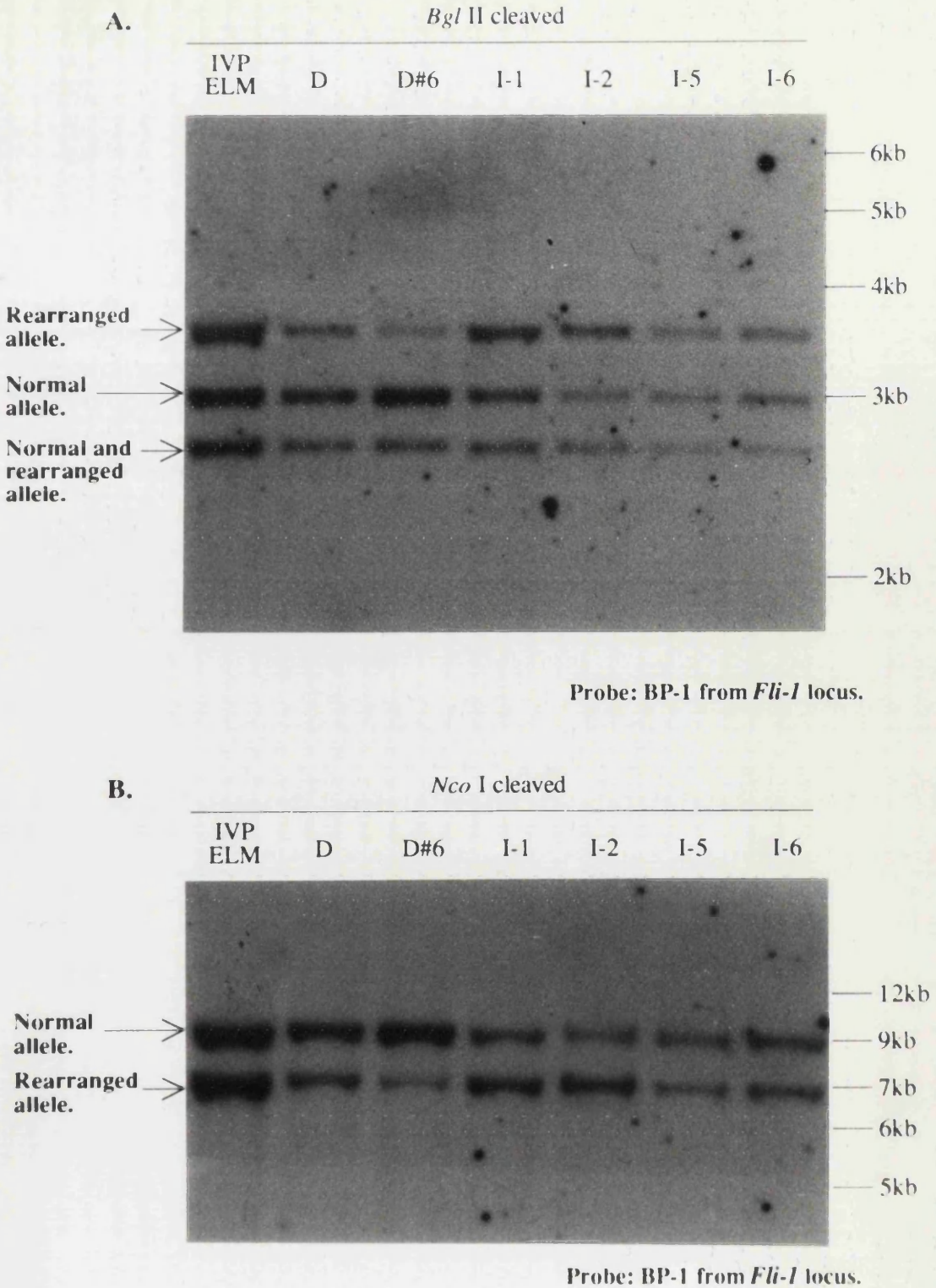

A.
Bgl II cleaved
IVP ELM
D
D#6
I-1
I-2
I-5
I-6
6kb
5kb
4kb
3kb
2kb
Rearranged allele.
Normal allele.
Normal and rearranged allele.
Probe: BP-1 from Fli-1 locus.
B.
Nco I cleaved
IVP ELM
D
D#6
I-1
I-2
I-5
I-6
12kb
9kb
7kb
6kb
5kb
Normal allele.
Rearranged allele.
Probe: BP-1 from Fli-1 locus.

Figure 12.8.

Figure 12.9: Southern blot analysis of *Nsi* 1-cleaved DNA suggests that there is a full-length retrovirus inserted at the *Fli-1* locus, whose structure remains unchanged throughout the ELM system.

Genomic DNA was isolated from various cell lines and tissues, cleaved with *Nsi* I and a Southern blot prepared according to Methods and Materials. The blot was first hybridised to ^{32}P-labelled BP1 probe derived from clone A (Figure 12.2) and exposed to X-ray film for four days (panel **A**) The position of molecular weight DNA markers electrophoresed adjacent to the DNA samples are shown to the right of the autoradiographs. The position of the '**normal allele**' and '**rearranged allele**' bands are labelled. The difference in the intensity of the bands between the ELM cell DNA lanes is due to variation in DNA loading: the differences apparent within a single lane (**D** and **D#6**) are due to the stromal cell contamination in these samples.
The blot was stripped and reprobed with a ^{32}P-labelled DNA fragment from the ***envelope*** gene of the F-MuLV retrovirus and exposed to X-ray film for four days (panel **B**). The '**common band**', which is present in all the samples, is labelled with a line shown at both sides of the autoradiograph. The position of molecular weight DNA markers electrophoresed adjacent to the DNA samples are shown to the right of the autoradiograph.

Tissues and cell lines examined were:
C3H spleen:- Normal spleen from a C3H mouse.
IVP ELM:- *In vivo* passaged ELM tumour cells.
D:- ELM-D cells growing on live MS-5 cells (ratio 4 ELM-D#6 cells per MS-5).
D#6:- ELM-D#6 cells growing on live MS-5 cells (ratio 6 ELM-D#6 cells per MS-5).
I-1, **I-2**, **I-5** and **I-6**:- Stromal cell-independent clones I-1, I-2, I-5 and I-6.

As mentioned in the text, when the two autoradiographs shown here are superimposed the '**rearranged allele**' band in panel **A**, and the '**common band**' in panel **B**, are coincident.

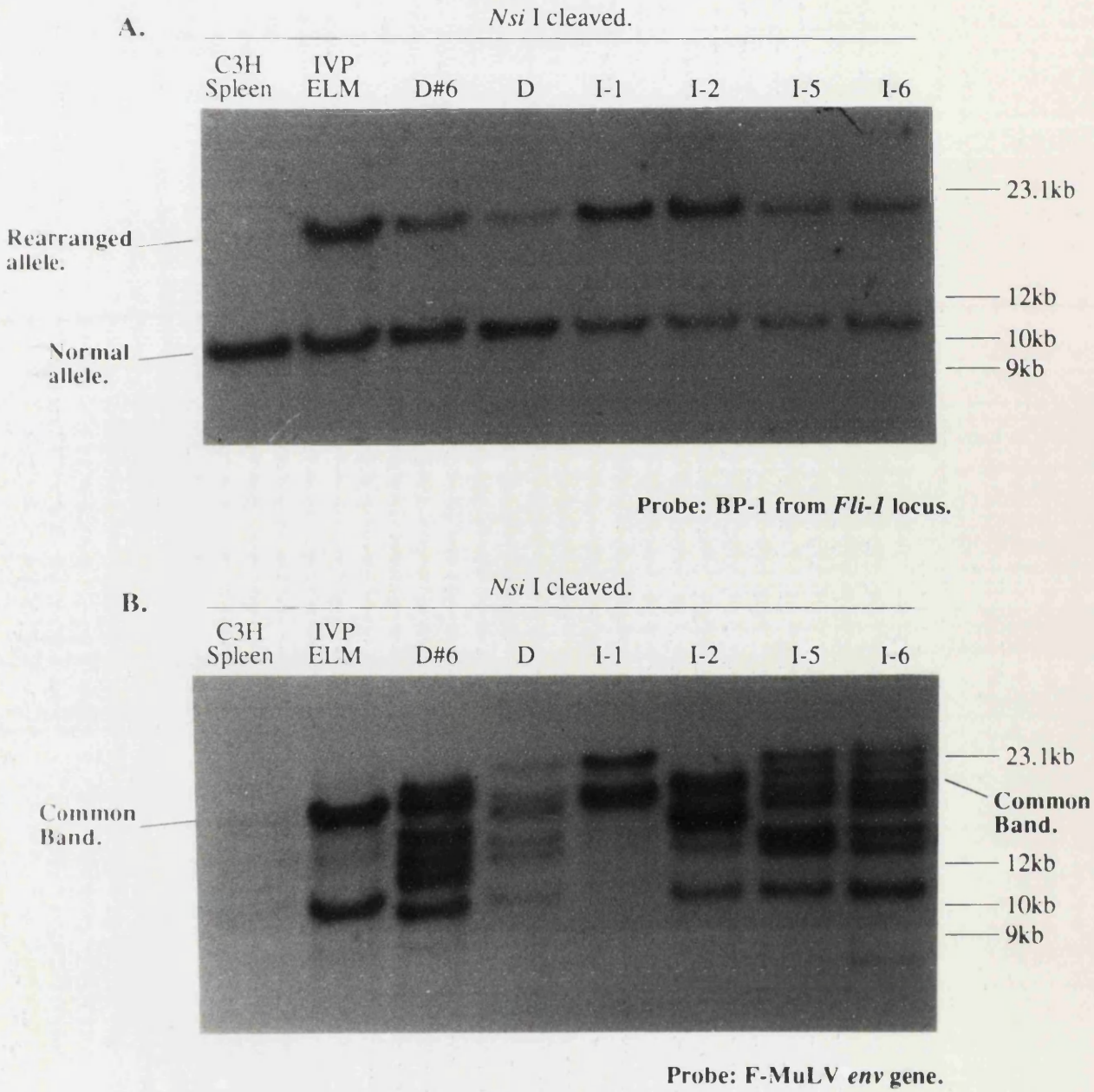

A.
Nsi I cleaved.
C3H Spleen
IVP ELM
D#6
D
I-1
I-2
I-5
I-6
Rearranged allele.
Normal allele.
23.1kb
12kb
10kb
9kb
Probe: BP-1 from *Fli-1* locus.
B.
Nsi I cleaved.
C3H Spleen
IVP ELM
D#6
D
I-1
I-2
I-5
I-6
Common Band.
23.1kb
Common Band.
12kb
10kb
9kb
Probe: F-MuLV *env* gene.

Figure 12.9.

cell lines derived from these original tumour cells, despite the fact that the *Fli-1* gene is only expressed in I-1 cells. No structural differences, detectable by Southern blot analysis, are apparent between the different ELM cell lines. Therefore, unlike the other integration events in the ELM tumour cells, the integrity of the provirus at the *Fli-1* locus appears to remain constant throughout the system. Possible explanations for all the phenomena described in this section are discussed in further detail in Part 4.

Figure 12.10: Restriction map of the *Fli-1* locus, with and without a full-length F-MuLV provirus.

The restriction map of the '**normal allele**' shown here has been constructed from known sites identified in 'phage clone A and from Southern blot analysis of C3H spleen DNA probed with the **BP1** probe (shown as a dark blue box). The hypothetical '**disrupted allele**' shown above this uses the restriction map for the normal allele and the map of the F-MuLV retrovirus (Perryman *et al*, 1991). Abbreviations used for the restriction sites are: **B** - *Bgl* II; **N** - *Nco* I; **Nsi** - *Nsi* I. The location of the **F-MuLV *env*** probe in the retrovirus is indicated with a dark blue box. The direction of transcription initiation mediated by the retroviral LTRs are represented by the arrows above the black boxes. The position and direction of transcription of the **first exon of the *Fli-1*** gene is also indicated in the normal and disrupted maps.

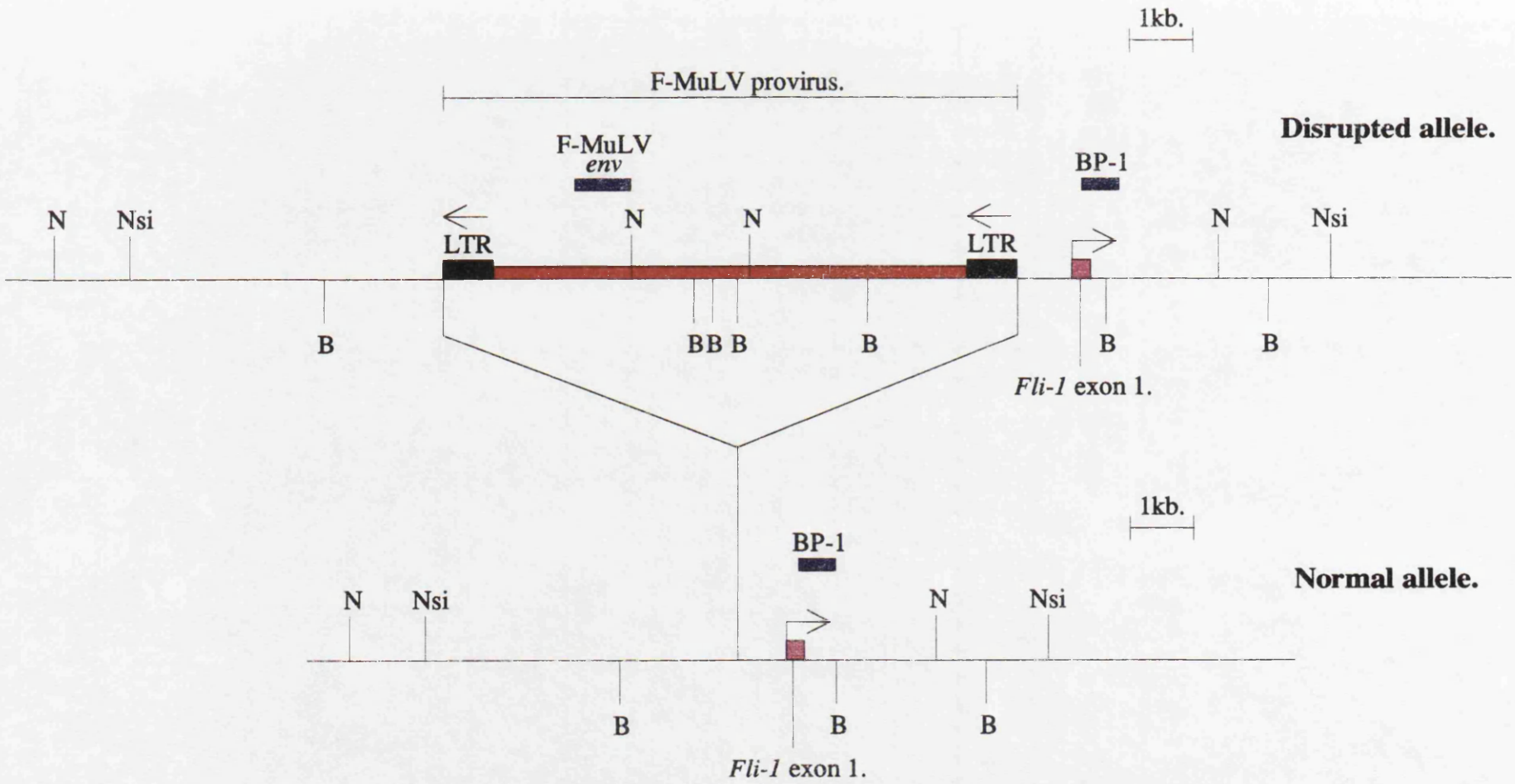

1kb.
F-MuLV provirus.
Disrupted allele.
F-MuLV
env
BP-1
N
Nsi
N
N
N
Nsi
LTR
LTR
B
B B B
B
B
B
Fli-1 exon 1.
1kb.
BP-1
Normal allele.
N
Nsi
N
Nsi
B
B
B
Fli-1 exon 1.

Figure 12.10.

PART 4: DISCUSSION.

CHAPTER 13: General Discussion.

13.1: The ELM system as a model for normal and malignant erythropoiesis.

The ELM erythroleukaemia is a very attractive model in which to investigate erythropoiesis and erythroleukaemia, initially for the following reasons. First, its origins appeared to be unique as it was reportedly induced with X-ray irradiation, which usually generates leukaemias derived from other blood cell lineages (Janowski and Boniver, 1986; Mole, 1986; Itoh *et al*, 1988a). There was no detectable endogenous or exogenous retroviral involvement, as assayed by reverse transcriptase activity and leukaemogenic virus production (Itoh *et al*, 1988a), unlike the previously described Friend virus- and radiation-induced leukaemias (Janowski and Boniver, 1986). Secondly, unlike the retrovirally-induced models it generated cell lines (ELM-D and its clonal derivatives, ELM-D#6, ELM-D#2 and ELM-D#3) which appeared to be much less advanced in terms of their loss of normal erythroid progenitor characteristics: that is, they required direct contact with a bone marrow-derived stromal cell layer to maintain long-term viability and prevent cell death (Itoh *et al*, 1988b, 1993). Identifying the growth factors and regulatory molecules produced by the stroma, and defining their role in supporting the growth of ELM-D cells, has obvious implications in our understanding of the control of normal and malignant erythropoiesis and the nature of stroma/progenitor cell interactions. However, the use of such tissue culture cell lines obviously requires that the interacting cells can proliferate in culture. This may cause a problem in terms of the relevance of the results to the *in vivo* situation, if in fact one function of stroma is to keep haemopoietic progenitor cells dormant and provide long-term maintenance until demand for a specific cell type arises. Clearly such an *in vitro* proliferating haemopoietic cell/stromal cell system may have lost an important aspect of the normal function. Nevertheless, the work presented in this thesis concerning SCF, along with the extensive studies performed by our collaborators in Dr. Ostertag's group (Itoh *et al*, 1993), have begun to reveal the complexity of these interactions in

controlling cell growth. Furthermore, this work demonstrated that mutant clones arose from these ELM-D cell lines, at a rate consistent with a mutational event, which had lost their stromal cell-dependence and were able to proliferate indefinitely in serum-containing medium alone. Defining the mutations responsible for this transition should give an insight into the nature of the intracellular signalling pathways which mediate the stromal cell-derived survival messages, and possibly further our knowledge of erythroleukaemia progression in the mouse. Significantly, the work described in this thesis has strongly implicated the ets family of transcription factors in preventing the cell death observed in ELM-D cells after separation from stroma.

13.2: Biological characterisation of the ELM system.

The initial experiments identified other phenotypic differences between the various cell lines of the system, namely their sensitivity to physiological differentiation inducers and their tumorigenicity in immunosuppressed syngeneic mice. All the cell lines tested, except ELM-I-1, could be induced to express *globin* mRNA and produce haemoglobin by incubation with erythropoietin, either alone, or in combination with IL-3, an effect which appears not to be due to differences in the expression of the *erythropoietin receptor* gene. The same differences in differentiation were observed *in vivo*, with only the ELM-I-1 cell line causing obvious anaemia, and an accompanying splenomegaly, upon injection. However, the ELM-I-5 cell line also induced a rapid splenomegaly, usually to a greater extent than ELM-I-1, but the cells were still capable of differentiation. Thus, the ELM cell lines can be categorised into four distinct phenotypic classes: the differentiation-sensitive, stromal cell-dependent cells and stromal cell-independent cells that (a) differentiated and had low tumorigenicity (ELM-I-2 and ELM-I-6), (b) differentiated, but had a much higher tumorigenicity (ELM-I-5), or (c) were differentiation-resistant *in vitro* and *in vivo* and also highly tumorigenic (ELM-I-1). Interestingly, the ELM-I-1 cell line shares all the characteristics attributable to Friend virus-induced erythroleukaemia cell lines

(section 2.3.4). Thus, by identifying correlations between particular mutations and certain phenotypes, and then demonstrating an effect of the mutation by introducing it into a phenotypically less advanced cell type, it should be possible to answer a number of questions concerning murine erythroleukaemias.

13.3: The role of SCF in stromal cell-associated erythropoiesis.

The acute anaemia observed in *Steel* and *W* mice, and the demonstration of a potent synergistic effect on erythroid 'burst' formation exerted by soluble SCF in combination with other growth factors, has strongly implicated this protein in stromal cell-derived stimulatory signals acting during erythropoiesis (see Introduction section 1.3.6). It was therefore an obvious candidate to study in the ELM-D#6/MS-5 coculture system, and in fact a form of *SCF* mRNA, which contains the region encoding the proteolytic cleavage site, is highly abundant in MS-5 cells. This results in the production of both the soluble and transmembrane (TM) form of the ligand (Itoh *et al*, 1993). On the other hand, the ELM tumour and all *in vitro* cell lines tested express the SCF receptor gene, *c-kit*, and interestingly, the evolution of the original ELM leukaemia appears to have resulted in a selective expansion of cells expressing this gene.

Soluble SCF is unable to permit the long-term growth of ELM-D#6 cells in the absence of stromal cells (Itoh *et al*, 1993), but it seemed possible that the TM form of the ligand could be a crucial part of the microenvironment established by the stromal cells, and required by ELM-D#6 cells. To test this, Ostertag and his group attempted to block the SCF/c-kit interaction by incubation with antibodies raised against the ligand and the receptor. These antibodies did not prevent the adherence of the two cell types, but did significantly reduce the growth of ELM-D#6 cells (>75%) compared to untreated controls (Itoh *et al*, 1993). These results suggested that TM SCF was indeed a necessary component of the stroma, a conclusion confirmed by experiments in which the MS-5 cells were replaced with defective stromal cells derived from a *Steel-Dickie* (Sl/Sl^d) mutant mouse. These mice are unable to produce

the transmembrane form of the ligand, although the soluble form is present at levels equivalent to wild-type animals (Flanagan *et al*, 1991; Brannan *et al*, 1991; Huang *et al*, 1992). The ELM-D#6 cells are unable to survive longer than a few weeks on these stromal cells, exhibiting similar properties to erythroleukaemic cells grown in soluble SCF without MS-5 cells (Itoh *et al*, 1993). Whilst it remains to be demonstrated that this effect is solely due to the absence of TM SCF, the result emphasises the importance of this alternative form of the ligand.

In summary, ELM-D#6 cells, despite being leukaemogenic, still have a requirement for TM SCF/c-kit interaction for optimum long-term growth *in vitro*; abrogating this interaction reduces viability and induces cell death, even in the presence of other erythropoietic growth factors. Whether TM SCF alone is sufficient for ELM-D#6 cell survival, remains to be tested. Other interactions established between the two cell types may also be involved, and even though IL-3, GM-CSF and epo are not produced by the stromal cells (Itoh *et al*, 1993), other erythropoietic regulators or components of the extracellular matrix may play a part in preventing ELM-D#6 cell death (see chapter 1 in the Introduction). This hypothetical combinatorial stimulation is perhaps analogous to the situation with mature T cells in which stimulation through CD4, CD8 and the TCR-CD3 complex cooperate to elicit maximal proliferation (reviewed in Rudd, 1990). However, it is possible that TM SCF may exhibit different properties than the soluble form which enable it to permit long-term viability. Indeed, this has been proposed by other workers (Dolci *et al*, 1991; Steel *et al*, 1992; Toksoz *et al*, 1992) who find that soluble SCF, unlike the TM form, is restricted in its ability to support the long-term viability of several SCF-responsive cell types. Moreover, this would explain the typical *Steel* phenotype of *Steel-Dickie* mice that appear to lack only the TM form (Flanagan *et al*, 1991; Brannan *et al*, 1991; Huang *et al*, 1992). However, recent unpublished data from Prof. Ostertag's laboratory suggests that under certain conditions long-term viability of ELM-D#6 cells can be supported, albeit less efficiently, by stromal cells derived from homozygous *Steel* (*Sl/Sl*) embryos which produce no SCF at all. It is not yet

known what factors these embryonic cells produce which support ELM-D#6 growth in the coculture. However, in conjunction with the results from the *Sl/Sl*^{d} stroma, this observation has led to the tentative hypothesis that TM SCF is required to provide a fine tuned balance between cell survival, and cell death induced by soluble factors such as soluble SCF. Such a hypothesis presupposes that different intracellular signals are elicited through the same receptor upon binding to slightly different forms of ligand. Experiments are being initiated to test this idea, employing expression vectors carrying the various forms of SCF to attempt to change the nature of SCF production in *Sl/Sl* stromal and their subsequent efficiency at supporting long-term ELM-D#6 cell viability.

The stromal cell-independent mutants have lost the requirement for stromal contact, but this is due neither to the endogenous production of SCF by these cells, nor to *v-kit*-like truncations of the SCF receptor (Besmer *et al*, 1986). However, a mutation in an ELM-D cell which mimics the stromal cell-derived signal would presumably be sufficient to permit the stromal cell-independent growth of that cell. Bearing in mind the dominant effect of IL-3, GM-CSF and epo, which induce clonal extinction or differentiation respectively even when this survival signal is continually present in a ELM-D#6/MS-5 coculture (Itoh *et al*, 1993), it remains a possibility that these growth factors could elicit the same effect on ELM-I cells containing the aforementioned mutation. This is the case with epo since all the cell lines, except ELM-I-1, differentiate. Very preliminary data suggest that ELM-I-2 undergoes a considerable reduction in cell proliferation in response to IL-3, unlike the highly tumorigenic ELM-I-1 and ELM-I-5 cell lines: this can not be adequately explained in terms of variations between the cell lines with respect to the extent of differentiation induction (see Figure 8.2 in the Results section). Although this observation requires confirmation, it may represent a further phenotypic difference within the ELM system, and possibly provide a clue as to the nature of the mutation responsible for stroma-independence in the various ELM-I mutants, i.e. ELM-I-2 may have constitutively activated the stromal cell-stimulated survival pathway(s), whilst ELM-

I-1 and ELM-I-5 may contain mutations which prevent programmed cell death at a point further downstream that can not be overcome by IL-3. One would assume that the ELM-I clones isolated in the presence of GM-CSF, but not dependent on GM-CSF for survival (see Figure 5.1 in the Introduction) would fit into the latter category.

13.4: Mutation of the *p53* gene in murine erythroleukaemias.

wtp53 is absent from all the erythroleukaemias arising after infection with Friend retroviruses (Ben David and Bernstein, 1991), and the ELM erythroleukaemia is no exception. Only one allele remains in all the ELM cells, and this contains an 18bp in-frame deletion which removes amino acid residues 206 to 211, inclusive, from the mature protein. The mutation is outside the previously defined 'hot-spots' identified by analysis of a wide variety of *p53* mutations, but small deletions and insertions have been reported in this region (Hollstein *et al*, 1991; Caron de Fromentel and Soussi, 1992). Interestingly, adjacent to the breakpoints of the deletion is a direct repeat of the sequence ACAG: similar repeats are seen in most other deletion mutations identified in the *p53* gene (Jego *et al*, 1993), and probably play an important part in the mechanism governing these types of mutations. The 18bp deletion removes part of the epitope recognised by the PAb 240 antibody which becomes exposed in a large number of p53 mutants that have lost their suppressor function (Gannon *et al*, 1990; Stephen and Lane, 1992). The fact that these mutations, which are dispersed throughout the gene, alter the conformation of this central region of the protein, implies that a mutation within this region would also result in the loss of wt properties. Therefore, although it has not been confirmed by transformation assays, it is highly likely that the mutant p53 protein present in ELM cells is unable to perform its normal functions.

Cells containing this mutant still undergo cell death in the absence of stromal cells *in vitro* and are able to differentiate in response to erythropoietin. The mutation is, therefore, unable to block differentiation or cell death. These observations appear to conflict with several recent publications that demonstrate apoptotic induction in

various cell types by the reconstitution of wt*p53* expression (Yonish-Rouach *et al*, 1991; Shaw *et al*, 1992; Ryan *et al*, 1993; Johnson *et al*, 1993). Of particular relevance, is the fact that when p53-negative Friend cells are transfected with wt*p53* expression vectors their cell cycle arrests at the G1/S boundary, apoptosis is induced exhibiting the distinctive characteristic of DNA fragmentation, and there is a marginal increase in the level of spontaneous differentiation (Ryan *et al*, 1993; Johnson *et al*, 1993). The differentiation induction in this case, and in other examples of wt*p53*-induced maturation (Feinstein *et al*, 1992; Shaulsky *et al*, 1991), may simply be the consequence of cell cycle arrest, since many chemical inducers of leukaemic cell differentiation block cell cycle progression by interfering with DNA replication (for example, the DMSO-induced differentiation of Friend cells (Terada *et al*, 1977)). Interestingly, the viability of wt*p53*-expressing Friend cells is restored by the addition of epo and differentiation returns to the normal background level (Johnson *et al*, 1993). Epo is known to prevent cell death in normal erythroid progenitors which then progress to form mature erythrocytes (Koury and Bondurant, 1990), but in Friend cells, even those expressing wt*p53*, other mutations involved in the development of the leukaemia probably contrive to inhibit differentiation. These wt*p53* reconstitution experiments led their authors to conclude that this gene induces death in erythroid progenitors, and therefore that *p53* mutations in developing erythroleukaemic cells prevents this process and increases their life-span. However, ELM-D#6 cells undergo cell death in the absence of MS-5 cells, even though they lack wtp53 proteins.

This paradox can be explained by assuming there are two forms of cell death in erythroid progenitors: one, wtp53-dependent, exhibiting classical apoptotic characteristics; the other, wtp53-independent, inhibited by stromal cell interaction. The latter process may occur by a separate mechanism and may be the reason why it has been difficult to demonstrate DNA fragmentation in ELM-D#6 cells (Ostertag, personal communication). Furthermore, this would explain the normal erythropoietic proliferation, cell death and differentiation that occurs in *p53*-null mice (Donehower *et al*, 1992). The current working model for p53 as a DNA damage-regulated

proliferation inhibitor (Lane, 1992; Figure 4.2 in the Introduction) has proposed that the wtp53 protein induces cell death only when the genome is disrupted by mutagenic agents. This would suggest that mutation of the *p53* gene in developing erythroleukaemia cells has no direct influence on cell proliferation or differentiation *per se*, but rather that it permits the survival of cells that have undergone DNA damage, a consequence of which will be the accumulation of oncogenic mutations and the loss of other tumour suppressor genes. It is these secondary mutations which are the probable cause of differentiation arrest and immortality. When wt*p53* expression is reconstituted in these Friend cells (Ryan *et al*, 1993; Johnson *et al*, 1993) this damage is detected, the cell cycle arrests and apoptosis is induced. In ELM-D#6 cells, cell death is the consequence of growth/survival factor deprivation and not a damage-induced apoptosis. One would hypothesise that this latter process would occur even in the presence of wt*p53* gene expression in normal erythroid progenitors, and thus may be more relevant to the production of erythrocytes and the progression of erythropoiesis, than the protective death induced by the wt*p53* expression.

13.5: The role of *ets*-related genes in the ELM system.

13.5.1: Expression of *Spi-1* and *Fli-1*.

Previous studies on Friend virus-induced erythroleukaemia cell lines demonstrated that if the original tumour was induced with SFFV or the Rauscher virus then the *Spi-1* gene became activated (Moreau-Gachelin *et al*, 1988, 1989, 1990), whereas use of the F-MuLV retrovirus caused deregulated expression of the *Fli-1* gene (Ben-David *et al*, 1990a, 1991; Sels *et al*, 1992). In the ELM system, which has putative F-MuLV involvement, *Spi-1* mRNA is only detectable using sensitive reverse transcription-PCR protocols and the protein is undetectable in EMSAs. This low level probably represents the expression seen in normal erythroid progenitors which has been reported by other groups (Tsai *et al*, 1991b; Galson *et al*, 1993). *Fli-1*, on the other hand, is expressed only in the ELM-I-1 cell line, the only

ELM cell line which is unable to differentiate in response to epo. In fact, this cell line is phenotypically very similar to the *Fli-1*- or *Spi-1*-expressing Friend cell lines. This supports the hypothesis that deregulated expression of *Fli-1* blocks erythroid differentiation: the ELM system presents an ideal opportunity to test this idea by ectopically expressing the gene in either other ELM-I cells or in the stromal cell-dependent clones, all of which are genetically very similar to the ELM-I-1 cell line. The lack of *Fli-1* mRNA in all the other ELM-I cell lines suggests it is not necessary for stromal cell-independent growth.

The ELM system maintains the correlation of *Fli-1* expression with F-MuLV infection, with *Spi-1* deregulation restricted to SFFV-transformed cells, observations which Ben-David and Bernstein (1991) have suggested may be due to differences in the structure of the LTRs of the two viruses. Both contain putative ets binding sites, and it seems plausible that one effect of *ets* gene deregulation may be the potentiation of retroviral expression. This would increase production of SFFV and F-MCF envelope proteins which are known to bind to, and constitutively activate, the epo receptor in the absence of ligand (Ruscetti *et al*, 1990; Li *et al*, 1990; Li and Baltimore, 1991), and could therefore be viewed as an advantageous step in leukaemic progression. Interestingly, the F-MuLV *envelope* gene is expressed to a much higher level in the *Fli-1*-expressing ELM-I-1 cells compared to the other ELM cells. However, a recent publication demonstrates, paradoxically, that Spi-1, but not Fli-1, is able to bind to the F-MuLV LTR (Zhang *et al*, 1993).

13.5.2: *Erg* expression and stromal cell-independence.

The presence of proteins in nuclear extracts from the ELM system that are able to bind to the E74 oligonucleotide in EMSAs, instigated an investigation into the expression of *erg*, an *ets*-related gene highly homologous to *Fli-1* which, when translated *in vitro*, codes for an E74 binding protein (Ben-David *et al*, 1991; Reddy and Rao, 1991). A portion of the previously undescribed murine *erg* gene was generated by reverse transcription-PCR reactions using primers designed from the

human gene (Rao *et al*, 1987) and the fragment sequenced. This demonstrated that (a) the protein is strongly conserved between species (only four amino acids out of 142 vary in the region analysed), (b) the ~85 amino acid ets domain, the putative DNA binding portion of the protein, is nearly identical to the equivalent region of the Fli-1 protein (three amino acids vary in a cluster of serine residues), and (c) the *erg* gene is probably subject to alternative splicing in a region adjacent to the ets domain, which plays an important role in other ets proteins in regulating DNA binding and transactivation properties (Janknecht and Nordheim, 1992; Rao and Reddy, 1992b; Dalton and Treisman, 1992; Pongubala *et al*, 1992; Wasylyk *et al*, 1992). Duterque-Coquillaud *et al* (1993) have very recently shown that this alternative splicing event is also seen in the human *erg* mRNA, and contributes to the formation of at least five transcript variants.

Analysis of the ELM system, using the *erg* cDNA fragment to probe Northern blots, demonstrated that whilst *erg* mRNA was very low in *in vivo* passaged tumour cells and three separate stromal cell-dependent clones, there was a significant (4-48-fold) up-regulation in all the fourteen stromal cell-independent cell lines. Mixing experiments have shown that this difference can not be explained simply by contaminating stromal cells (*erg* mRNA-negative) diluting the *erg*-hybridising signal on the blot, although this of course will contribute slightly to the observed changes. Interestingly, expression of the *erg* gene appears to be subject to stromal cell regulation in the ELM-D#6 cell line, whereas this is lost in the ELM-I cells. This is implied by the fact that ELM-D#6 cells separated from stroma have a 5-10-fold increased level of erg mRNA . (These pure populations of ELM-D#6 cells are viable for between one to two weeks in the absence of stroma, but then undergo cell death). However, when the cell lines ELM-I-2 and ELM-I-5 are allowed to adhere to MS-5 cells and grow in coculture for 72 hours, the *erg* gene is *not* down-regulated. Thus, the expression of the gene in the ELM-D#6 cells may be an essential component of a response to loss of stromal cell contact which temporarily elicits an ELM-I-like phenotype on the cells, and allows short-term survival. However, the cells will

eventually undergo cell death, unless the pathway becomes constitutively active. One way this could possibly happen is by direct deregulation of the *erg* gene as a result of a mutational event, such as proviral insertion, and whilst I have been unable to detect either rearrangement or amplification at the *erg* locus in four ELM-I cell lines, this may be a consequence of only a partial cDNA probe being available at present. Further analysis using a full-length cDNA may reveal these types of mutations: cDNA library screening is underway to isolate such a probe. This would also allow the hypothesis that *erg* alters stromal cell-dependence to be tested by its ectopic expression in ELM-D cells.

In summary, there is a correlation between expression of the *Fli-1* gene and a block in erythropoietin-induced differentiation, and *erg* expression and stromal cell-independent survival and proliferation. Future experiments designed to verify the validity of these observations are discussed in chapter 14.

13.5.3: Detection of ets proteins in the ELM system.

At the outset of this part of the project, erythroleukaemia cell lines were screened for the expression of ets proteins by using three oligonucleotides, each known to bind to a subset of different ets proteins. With ELM-I-1 and ELM-I-2, no detectable binding was apparent with the PU-1 and PEA3 oligonucleotides, thus effectively excluding Spi-1, c-ets-1 and c-ets-2, a conclusion confirmed by Northern blot analysis. However, a complex pattern of bands was observed when the E74 oligonucleotide was used: erg had previously been shown to recognise this sequence (Reddy and Rao, 1991) and gene expression was subsequently analysed. The homology between erg and Fli-1 over their putative DNA binding domains prompted experiments to examine the DNA binding specificity of Fli-1 protein produced in a reticulocyte translation system. In fact, this protein exhibited similar specificity as erg, a result confirmed by Zhang *et al* (1993), who extended the studies to demonstrate a modest increase in the transcription of a reporter gene linked to tandem arrays of the E74 oligonucleotide co-transfected with a *Fli-1* expression vector. The

E74 binding activity in the ELM-I-1 and ELM-I-2 extracts could, therefore, conceivably be due to the Fli-1 and erg proteins, although this can only be confirmed by performing 'super-shift' EMSAs with Fli-1- or erg-specific antisera. However, the E74 oligonucleotide must be able to bind to other ets proteins. First, MS-5 cells which are *erg*-negative and only express *Fli-1* at very low levels, give retarded bands with E74 some of which have identical mobility and are subject to the same pattern of competition with other ets binding sites as those seen with ELM-I-1 and ELM-I-2. Second, comparison of ELM-D#6 EMSAs with the ELM-I results identifies few, if any, significant differences in the bands formed, despite the obvious differences apparent at the mRNA level with respect *Fli-1* and *erg* expression. These results have led to two tentative conclusions: (a) other E74-binding ets-related protein(s) are probably expressed in the ELM system, the identification of which may be important in understanding the role of deregulated *ets* genes in erythroleukaemia as these may interfere with processes controlled by other ets proteins; and (b) DNA binding specificity and affinity determined using *in vitro*-produced ets proteins may not necessarily mirror the *in vivo* situation. Bearing in mind that the sequences recognised by other ets proteins are altered by post-translational modifications and hetero-oligomerisation, this is not a trivial point. However, this presupposes that the *Fli-1* and *erg* transcripts are translated in ELM-I-1 and ELM-I-2 cells, which needs to be confirmed with antibodies when they become available.

13.6: The effect of retroviral integration at the *Fli-1* locus in the ELM system.

The restriction of *Fli-1* expression to the ELM-I-1 cell line suggested that transcription of this gene had become deregulated, possibly as the consequence of a mutational event. Using a genomic probe from the *Fli-1* locus, and a probe from the 5' end of the *Fli-1* cDNA, a rearrangement was detected in one allele in the DNA of ELM-I-1 cells. Cloning the rearranged *Fli-1* allele revealed that the rearrangement was caused by the insertion of a F-MuLV-like provirus at a position identical to those

previously described by Ben David and his colleagues (1990a, 1991); that is, upstream of, and in the opposite orientation to, the *Fli-1* gene.

The sequence data presented here from the *Fli-1* locus has defined the position of the putative first exon of the gene, which in the ELM cells is approximately 900bp from the proviral insertion site. This exon contains the previously defined 5' non-coding portion of the gene and the first six codons (Ben David *et al*, 1991). Interestingly, the Cas-Br-E integration sites identified by Bergeron et al (1991) all appear to lie within this first exon, and unlike the F-MuLV integrations are in the same transcriptional orientation as the gene: this has recently been confirmed (Bergeron *et al*, 1992). Thus, it seems likely that *Fli-1* is activated by two distinct mechanisms, depending on the retrovirus used to induce the leukaemia; enhancer insertion with F-MuLV, but transcriptional read-through with Cas-Br-E. The preliminary analysis of the *Fli-1* gene also identified a dinucleotide repeat ($(AG)_{23}$) just upstream of the start of the first exon. The exact role of this sequence, if any, in the transcriptional regulation of *Fli-1* is not known, but it has been suggested that formation of left-handed double-helical Z-DNA by these sequences may increase the rate of formation of unwound DNA required during transcriptional initiation (reviewed in Hill, 1991). Moreover, it has also been proposed that they act as targets for recombination and insertion events, and it is tempting to hypothesise that the dinucleotide repeat at Fli-1 may play a part in retroviral integration at this locus in haemopoietic cells.

An intriguing and novel aspect of this work was the detection of the F-MuLV integration at *Fli-1* in the *in vivo* passaged tumour cells and all the derivative cell lines, despite the restricted expression of the gene to the ELM-I-1 cells. This raises important questions concerning (a) the role of F-MuLV integrations at the *Fli-1* locus in the ELM cells and other erythroleukaemia cell lines, and (b) the mechanism whereby *Fli-1* becomes expressed in ELM-I-1 cells. Currently, there is no definitive answer to either of these questions, but several hypotheses will be considered, some of which are supported by experimental observations.

The fact that the tumour cells are monoclonal with respect to this insertion implies that it was selected for during tumour development. There are precedents in other systems for such insertions being selected due to activation or inactivation of adjacent genes (reviewed in Peters, 1990). However, in the ELM leukaemia inactivation seems unlikely since there is no evidence for *Fli-1* expression in normal erythroid progenitors, and furthermore, only one allele appears to be affected by mutation in the ELM tumour. Also, other work with a number of independently-isolated leukaemia cell lines, strongly implies that insertions at the *Fli-1* locus deregulate expression of the *Fli-1* gene (Ben-David *et al*, 1991; Bergeron *et al*, 1992). There seem to be two plausible interpretations of the ELM data involving gene activation by retroviral insertion. First, *Fli-1* could have been activated in the original tumour but then become inactivated during subsequent passage *in vivo*, i.e. *Fli-1* expression was required for initiation of the leukaemia but not for its subsequent maintenance. However, if this hypothesis is correct, inactivation of *Fli-1* must have occurred very early since tumour cells obtained from the very first passage of the original leukaemic spleen only express levels of *Fli-1* mRNA consistent with contamination by normal spleen cells. An alternative explanation is that the retroviral insertion upstream of the *Fli-1* gene activates the expression of another gene nearby, and it is the expression of this gene which encourages leukaemic progression. *Fli-1* is known to be linked to *c-ets-1* (Ben David *et al*, 1991), but this is not expressed in the ELM tumour or its derivative cell lines (data not shown). However, it remains possible that other genes in the vicinity of the integration site become ectopically expressed and are advantageous in tumour progression: possible candidates are the mouse homologues of *PLZF* and *ALL1*, genes closely related to known *Drosophila* developmental genes and which are the targets for translocations involving 11q23 in human leukaemias and lymphomas (Tkachuk *et al*, 1992; Gu *et al*, 1992; Djabali *et al*, 1992; Ziemin van der Poel *et al*, 1992; Chen *et al*, 1993). (Human 11q23 is syntenic with the position on mouse chromosome 9 where *Fli-1* and *c-ets-1* reside).

The second question concerns the mechanism whereby *Fli-1* becomes expressed in ELM-I-1. No structural differences have been detected between ELM-I-1 and other ELM cell lines using Southern blot analysis of the *Fli-1* locus, although it can not be excluded that small sequence changes, in either the normal or the rearranged alleles, or other alterations outwith the region screened in this study, may be affecting expression of the *Fli-1* gene. Alternatively, ELM-I-1 may express transcription factors absent from other ELM cell lines, that permit *Fli-1* transcription which again may be either from the rearranged or the normal allele. This putative alteration in the transcription factor profile of ELM-I-1 cells, possibly as a consequence of mutational events, may be responsible for the different phenotype of ELM-I-1 cells. One possibility is that the reported resistance of C3H mice to F-MuLV-induced neoplasms (Ruscetti *et al*, 1981) may be due to an inability of the LTR to be appropriately activated in the haemopoietic cells of this strain: expression in ELM-I-1 cells may reflect a loss of this genetic control. A third hypothesis is that DNA methylation at CpG dinucleotide sequences, a process often associated with the transcriptional silencing of cellular and viral promoters (Bird, 1992; Orend *et al*, 1991), inactivates *Fli-1* expression in all but the ELM-I-1 cells. Preliminary evidence derived from the comparative analysis of the banding patterns of Southern blots of DNA digested with *Msp* I (recognition sequence: CCGG) or its methylation-sensitive isoschizomer *Hpa* II, have suggested that several CpG sites around the breakpoint are hypomethylated in the ELM-I-1 cell line (data not shown). What effect, if any, these differences have on *Fli-1* expression is unknown: in any case, as is often the problem with these kind of studies, it is difficult to conclude whether hypermethylation is the cause, or the consequence, of gene silencing (Bird, 1992). However, it may be significant that the expression of F-MuLV sequences is significantly higher in the ELM-I-1 cells than in any of the other ELM cell lines, which suggests that the activity of the retroviral LTR varies between the cell lines and this in turn could explain the *Fli-1* expression differences. One way to distinguish between these possibilities would be to introduce an expression plasmid containing a reporter gene linked to the

F-MuLV LTR and *Fli-1* promoter (available from clone A) into the various cell lines of the system. If, for example, this reveals that only the ELM-I-1 cells are capable of transcription from such a promoter, it would imply that only these cells contain the appropriate transcription factor profile required to mediate *Fli-1* expression.

13.7: Radiation and retroviruses.

The work presented here questions the non-viral origin of the ELM leukaemia, since the *in vivo* passaged tumour cells, and all the cell lines derived from them, contain (a) expressed exogenous retroviral sequences which hybridise to the *env* gene of F-MuLV, and (b) genomic LTR and *gag* sequences nearly 100% homologous to the published sequence derived from a F-MuLV strain (Perryman *et al*, 1991). Furthermore, it is possible to observe viral particles budding from cultured ELM cells under the electron microscope (Ostertag, personal communication). Exactly when, and how, retroviral infection occurred remains a mystery, but the presence of retroviral sequences in the earliest *in vivo* ELM cells available suggests that it probably played a major part in the evolution of the tumour. Thus, although radiation may have been a contributory factor, it was almost definitely not the sole etiological agent. Interestingly, since F-MuLV has only been reported to induce leukaemias in newborn mice (Troxler and Scolnick, 1978; MacDonald *et al*, 1980; Oliff *et al*, 1981), and only in Balb/c and NIH/Swiss strains (Ruscetti *et al*, 1981), the radiation may have been necessary to encourage the progression of this erythroleukaemia in a C3H background, which did not appear until the mouse was over one year old (Itoh *et al*, 1988a).

A further complication arose when the number and structure of the F-MuLV proviruses was investigated by Southern blot analysis. By using the appropriate restriction endonucleases and a retroviral probe, junction fragments between the host DNA and the provirus were assessed. This revealed that not only did the number of proviruses vary from between two to nine in the ELM cells, but also that only one maintained its structural integrity throughout the system. As was discussed earlier,

this insertion appears to be at the *Fli-1* locus. The generation of new insertions may be the consequence of superinfection by F-MuLV retroviruses or recombinants produced either by the ELM cells, or by the MS-5 stromal cells, which also surprisingly express F-MuLV transcripts. (Like the ELM erythroleukaemia, the MS-5 cell line was reportedly generated by irradiation, in this case of a long-term bone marrow cultures (Itoh *et al*, 1989)). The apparent shifting of insertions, seen for example between the *in vivo* passaged tumour cells and the ELM-D cell line, may be a result of recombination events between F-MuLV and endogenous, or other F-MuLV, sequences present in these cells. However, there is little evidence to support any of these possible explanations which could only be effectively proven by cloning the proviruses from genomic libraries constructed from the DNA of the various cell types. Perhaps a more important goal is to determine whether the proviruses affect the structure or expression of tumour suppressors or proto-oncogenes. In particular, whilst it is known that one of the proviruses in the *in vivo* passaged tumour cells is inserted at the *Fli-1* locus, the position of the second is unknown: since the cells are monoclonal with respect to this integration event, it seems likely that it conferred some advantage to the developing ELM tumour. Furthermore, the novel integrations apparent in the ELM-I-5 and ELM-I-6 cells, but absent from the ELM-D#6 cell line from which they were derived, may have been important in the evolution of stromal cell-independence.

There is no evidence for the presence of the other component of the Friend virus complex (SFFV) in the ELM system, demonstrated by the fact that (a) gp55-hybridising sequences are expressed at only a very low level equivalent to that seen in normal C3H spleen cells, and (b) no novel insertions are detectable in the DNA of the *in vivo* passaged tumour cells. However, genomic DNA from ELM-I-5 contains a novel *gp55*-hybridising band arising not as the result of SFFV infection, but probably as a consequence of a genomic rearrangement within this cell line. Furthermore, using the same probe, Northern blot analysis suggested that the ELM-I-5 cells produce an increased amount of retroviral transcripts, and also produce a novel

transcript absent in the other ELM cell lines. The significance of these observations is at present unclear, and it remains to be determined whether they influence the biological properties of the ELM-I-5 cell line in any way. One possibility, is that this putative rearrangement may generate a tumorigenic retrovirus, in a manner akin to that seen in other F-MuLV-induced malignancies (Ruscetti *et al*, 1981; Chesebro *et al*, 1984). Li and Baltimore (1991) have demonstrated that these retroviruses behave like SFFV, expressing envelope proteins capable of ligand-independent activation of the erythropoietin receptor. If the ELM-I-5 cell line does express a recombinant retrovirus of this type, then it may explain the increased splenomegaly caused by these cells since host erythroid cells could develop epo-independence as a result of cross-infection. Such an effect is readily tested by determining the ratio of ELM-I-5 to host cells in the enlarged spleens which develop on ELM-I-5 injection.

CHAPTER 14: Future Prospects.

The data presented in this thesis have initiated two long-term avenues of research for subsequent study: (1) the elucidation of the molecular mechanisms involved in the critical stromal cell interactions which support long-term ELM-D growth, in particular the role of signalling through c-kit in response to binding to soluble or membrane-bound SCF; and (2) the investigation of the roles of the *erg* and *Fli-1* genes in generating abnormal phenotypes in erythroid progenitor cells and the effect this has on erythroleukaemia progression. The results from these studies should provide a working model concerning intracellular signalling in response to survival and differentiation stimuli.

With respect to SCF, retroviruses expressing the various forms of the protein should be useful tools in determining if they have distinct functional properties. Experiments already underway in Prof. Ostertag's laboratory involve the use of such retroviruses to alter the SCF expression in *Sl/Sl* and Sl/Sl^d stromal cells and assay for

changes in these cells to support long-term ELM-D growth. Alternatively, it may prove informative to infect the ELM-D cells themselves with these viruses to see if it increases the generation of stromal cell-independence. One possible problem with this experiment is that the expression of SCF, in particular the TM form, and c-kit in the same cell may prevent proper interaction. A different approach is to assess whether ELM-I mutants have mutations in c-kit that constitutively activate the receptor. This could be done by measuring the protein's kinase activity by assaying autophosphorylation after immunoprecipitation. Likewise, it may be interesting to introduce *v-kit* (Besmer *et al*, 1986) into ELM-D cells and again assess for alterations in the rate at which stromal cell-independent variants arise.

Crucial experiments to directly examine the putative role of *erg* and *Fli-1* in the phenotypic differences in the ELM system can be performed by constitutively expressing *Fli-1* or *erg* cDNAs in ELM-D or ELM-I cells. This will require the cloning of a full-length *erg* cDNA and the construction of expression vectors or retroviruses carrying the genes. Hypothetically, one might expect that *Fli-1* will block differentiation in these assays, whilst *erg* may increase the rate of generation of stromal cell-independent variants. However, bearing in mind the high homology between the two ets proteins (almost 100% over the ets domain) (Ben-David *et al*, 1991; Watson *et al*, 1992), it is possible that high ectopic expression of either gene in the ELM system may affect both survival *and* differentiation. The erythroid maturation observed in *erg*-expressing ELM-I clones, which were selected for stroma-independent growth rather than differentiation arrest, may be due to *erg* not being directly deregulated by mutation. In this way, epo may still override the constitutively active survival signal present in these cells, perhaps in a manner similar to the way that it overrides the stromal cell-derived signal. If *erg* or *Fli-1* were to induce phenotypic change in ELM cells, it would prove interesting to introduce partially-deleted or mutated versions of the genes into these cells to define the domains required, and also suggest possible mechanisms, for each particular phenotypic change observed. For example, cDNAs encoding only the DNA binding

domain of the proteins may act as dominant-negative inhibitors of Fli-1, erg or other ets proteins (such as the E74 binding protein identified in ELM-D cells).

The validity of any results obtained in these experiments would be reinforced by using erg and Fli-1 anti-sera to verify protein production. Further, these would allow the 'super-shift' experiments proposed in section 13.6.3 to be performed to compare the DNA binding specificities of these proteins when they are produced in *in vitro* translation systems as opposed to within live cells, where hetero-oligomerisation and post-translational modifications must be considered.

If up-regulation of *erg* and *Fli-1* are responsible for generating some aspects of the leukaemic phenotype, these effects should be manifest by expressing the proteins in normal haemopoietic progenitors. Using retroviral expression vectors, these genes can be introduced into haemopoietic cells and the growth factor requirements and differentiation properties assessed using standard protocols, such as BFU-E and CFU-E assays, with or without stromal cells. Generation of leukaemic cells could be determined by transplantation *in vivo* or by direct infection of mice with the retroviruses. Since the ELM leukaemia cells contain *p53* mutations, it might also prove informative to introduce these expression vectors into *p53*-null mice (Donehower et al, 1992) with the assumption that one genetic event which occurs during the progression of erythroleukaemias, is already present.

PART 5: REFERENCES.

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