A study of the transcriptional regulation of the defensin genes

This thesis is submitted in partial requirement for the Degree of Doctor of Philosophy at the University of Glasgow.

Andrew Christopher Phillips

The Beatson Institute for Cancer Research

December 1994

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Acknowledgements

I would like to thank all of those people at the Beatson who have provided help and advice, in particular my supervisor Dr. Birnie for help and encouragement throughout my time, and advisors, Dr. Plumb and Dr. Neil. In addition thanks to those in the group who both helped and put up with my mess - Marion, Tam, Elaine, Murdo, Richard and Viv *et al.* I am also most grateful to those with whom I discussed my work and science generally; Mat Grove, Chris Kemp, Chris Bartholomew, Scott Cuthill, Rob Nibbs and in particular Kev Ryan. I would also like to thank Tessa Holyoak for help in obtaining samples.

Most importantly I would like to thank my parents for support and encouragement throughout my education.

Abstract: A study of the transcriptional regulation of the defensin genes

The defensin genes are expressed almost exclusively within a narrow window of myeloid differentiation, namely at the myelocyte stage of granulopoiesis. These genes encode peptides which are involved in the granulocytes response to infection and inflammation, being capable of destroying a wide range of organisms. The limited expression pattern of these genes suggests they would provide a good model to study mechanisms of both myeloid and differentiation-stage-specific gene expression. By studying the regulation of these genes, it may be possible to identify transcription factors which control both lineage-specific gene expression and differentiation in the myeloid compartment. This information may provide an insight into the process of maturation arrest and leukaemogenesis.

Initial work was undertaken to identify a system in which to study the regulation of these genes. However, despite previous reports, no expression could be detected or induced in any myeloid cell line tested. DNAse1 hypersensitivity analysis was carried out on a range of primary samples and both myeloid and non-myeloid cell lines. This analysis identified a number of DNAse1 hypersensitive sites in the chromatin. One of these sites spanned the promoter and this was analysed in some detail. Both *in vitro* binding studies and transfection of reporter constructs were used to investigate if this promoter could mediate myeloid and/or differentiation-stage-specific activity. A number of DNA binding activities were identified which were markedly regulated during myeloid differentiation. This, in conjunction with the transfection data, allows models to be produced to explain both the myeloid and differentiation-stage-specific activity of these genes.

Evidence is provided implicating the defensin genes as targets for regulation by the transcription factor c-Myb. This factor acts through a composite element within the defensin promoter which also binds a C/EBP-like factor. In addition, Ets-like transcription factors are implicated in mediating the myeloid specificity of these genes.

Contents

Part 1: Introduction

List of figures

Part 1: Introduction

Chapter 1: Haemopoiesis

Haemopoiesis in the adult human takes place principally in the bone marrow and lymphoid organs and is responsible for the regulated production of blood cells. This is a continuous process with more than 10^{11} cells per day being generated in the average adult (reviewed in Dexter and Spooncer 1987). The haemopoietic system is conventionally classified into three compartments: stem cells, progenitor cells and maturing end cells. Stem cells are defined by their ability to reconstitute the entire haemopoietic system, and these are capable of both self renewal and differentiation with proliferation to generate the precursor cells. These cells undergo a further series of poorly understood differentiation steps to generate committed maturing end cells. These mature 'end' cells are comprised of erythrocytes, monocytes, neutrophils, eosinophils, megakaryoctes, mast cells, B lymphocytes and T lymphocytes. The characteristics of the differentiation of macrophages and neutrophils are described in more detail in sections 1.1(i) and 1.1(ii), as these cells are most relevant to the work described here.

1.1 (i) Monocytic differentiation

The monocytic/macrophage lineage develops initially in the bone marrow. Monocytes are released into the circulation where they later seed tissues and develop further into tissue macrophages. Monocytes are less common than neutrophils, making up 2-10 % of leukocytes in the peripheral blood.

The monoblast is the first cell defined in the monocytic lineage and is morphologically similar to the myeloblast. These cells are characterised by a round nucleus with fine chromatin and prominent nucleoli and a cytoplasm containing numerous mitochondria, show little motility or adhesiveness to glass surfaces. When these cells develop a complex golgi and a granule population they can be described with certainty as promonocytes. The promonocyte is characterised by a

relatively large diameter (10-20μm) and a high ratio of nucleus to cytoplasm. These cells have a conspicuous golgi complex, relatively few cisternae of the endoplasmic reticulum, numerous free polysomes and a large number of granules. Promonocytes have peroxidase activity, adhere to glass and can endocytose but show little phagocytosis.

Monocytes are found in the circulation and are smaller in size than promonocytes. The centrally located nucleus is indented or horse-shoe shaped, the cytoplasm contains many lyzosomal granules and have increased activity for a number of hydrolases. Monocytes further develop in tissues into the large macrophage cell . These macrophage are not end cells; immature macrophages are capable of division and give rise to long lived non-replicating macrophages. Maturation is accompanied by an increase in cell size and in numbers of cytoplasmic organelles including lyzosomes and mitochondria. The precise pattern of development is determined by the tissue in which the macrophage matures. This produces sub-populations of macrophage with distinct biochemical properties e.g. alveolar macrophage and peritoneal macrophages (for review see Williams *et al.*, 1986).

1.1 (ii) Neutrophil differentiation

The polymorphonuclear neutrophil (PMN) when released into the blood stream is a terminally differentiated cell, no longer capable of proliferation and with a limited capacity for protein synthesis. These PMNs account for around 50-60 % of circulating white blood cells, and have a relatively short life-span - circulating for hours before migrating to the tissues, where they normally live for 1-2 days before senescing.

The first cell described in the neutrophil lineage is the myeloblast; this cannot be reliably distinguished by morphological criteria from other primitive haemopoietic cells, and is normally identified by 'the company it keeps' i.e. the

surrounding cells in the marrow. This primitive cell is restricted to the bone marrow, contains a large nucleus which is round or slightly oval with one or more prominent nucleoli. The cytoplasm contains no granules and the endoplasmic reticulum is poorly developed. The promyelocyte is the first cell that can be reliably identified as a neutrophil precursor, and is characterised by a large round nucleus with diffuse chromatin and a large number of nucleoli. Often the nucleus is indented opposite the golgi apparatus and has a smaller nuclear/cytoplasmic ratio than the myeloblast. Characteristic of the promyelocyte is the accumulation of peroxidase positive granules (primary granules) in the cytoplasm and a more developed endoplasmic reticulum.

As the cells mature through the myelocyte and metamyelocyte stages the chromatin condenses and the nucleoli become less prominent and eventually disappear. The nucleus shrinks and the cells become progressively smaller. The endoplasmic reticulum is less developed than in the promyelocyte, polyribosomes are less abundant and mitochondria are smaller and fewer in number. A second population of granules appear (secondary or specific) and the manufacture of the primary granules ceases and are diluted with division until the secondary granules become predominant. Division takes place only during the myeloblast, promyelocyte and myelocyte stages. The mature PMN is characterised by a lobed nucleus and marked condensation of the chromatin. The endoplasmic reticulum is not well developed, and there are relatively few mitochondria and ribosomes in comparison to precursors - reflecting the diminished protein synthesis capacity of the mature PMNs (for review see Williams *et al.*, 1986).

1.1 (iii) Neutrophil function

Neutrophils are phagocytic cells whose primary function is the ingestion and destruction of invading micro-organisms. In the blood, neutrophils are attracted to sites of infection by chemotactic factors, which are generated by interaction of

antibodies with antigen or pathogens. These cells then ingest opsonized particles (antibody and complement coated) by surrounding them with pseudopodia which eventually meet and fuse to enclose the micro-organism within an intracellular vesicle termed the phagosome. The cytoplasmic granules then fuse and release their contents into this phagosome.

Neutrophils contain two types of granule; the primary (or azurophilic) and the secondary (or specific granules). Primary granules are similar to lysozmes i.e. membrane bound particles storing acid hydrolases in a latent form, but also contain a number of specialised microbicidal proteins not found in other tissues. These include myeloperoxidase, involved in the oxidative destruction of ingested particles and a number of cationic peptides which include the bactericidal / permeability inducing protein and the defensins which are microbicidal (see section 1.2).

While the primary granules are largely used intracellularly, the secondary granules are released into the extracellular space. Proteins contained in these granules include collagenase, plasminogen activator, lysozyme and lactoferrin. Collagenase and plasminogen activator are involved in the destruction of tissue prior to repair at sites of inflammation, lysozyme is present in the secondary as well as in the primary granules and both this and lactoferrin are involved in the destruction of micro-organisms.

1.2 Defensins

Defensins are small cationic, arginine-rich peptides of 29-34 amino acids in length. With the exception of the so-called β-defensins identified in cow PMN (Selsted *et al.*, 1993), all defensins contain an invariant six cysteine residue motif. The connectivity of their three disulphide bridges has been determined by direct chemical analysis (Selsted and Harwig 1989) and confirmed by 2D nuclear magnetic resonance (Pardi *et al.*, 1992). Defensins have been identified in the PMNs of human, guinea pig, rabbit, cow and rat but surprisingly seem to be

absent from mice (Eisenhauer and Lehrer 1992).

These molecules are found predominantly in the primary granules of neutrophils where they account for approximately 30% (Greenwald and Ganz 1987) of total primary granule protein. However these peptides have also been found in pulmonary macrophages of rabbits and related proteins have been identified in paneth cells of the mouse small intestine (Ganz *et al.*, 1989; Ouellette *et al.*, 1989). Homologous molecules have also been found in the haemolymph of certain insects after injury or infection (Lambert *et al.*, 1989).

Human and rabbit defensins are synthesised as a 93-95 amino acid preprodefensin with a characteristic 19 amino acid signal sequence probably necessary for insertion into the endoplasmic reticulum, and a 40-45 amino acid anionic propeptide which must be cleaved to yield the mature defensin molecule (Valore and Ganz 1992).

1.2 (i) Antimicrobial activities of defensins

Defensins have potent antimicrobial activity against a wide variety of both gram-positive and gram-negative bacteria, fungi, enveloped viruses and tumour cell lines. Four human peptides have been isolated termed HNP1, HNP2, HNP3 and HNP4. HNP1 and HNP3 are identical except for the N-terminal amino acid, and HNP2 lacks this amino acid. Only cDNAs corresponding to HNP1 and HNP3 peptides were isolated from an HL60 cDNA library suggesting that HNP2 may be derived by cleavage of HNP1 or HNP3 (Daher *et al.*, 1988). HNP4 was later purified from the granules of neutrophils (Wilde *et al.*, 1989). HNP4 contains the conserved cysteine motif, however it is considerably diverged from the other human defensins. Of the 33 amino acids, 22 and are different from HNP1, and many of these changes require more than a single nucleotide change indicating the genes encoding these peptides are not the result of a recent duplication event, as may be the case for the genes encoding HNP1 and HNP3.

Differential activities have been found for the HNP peptides against a panel of bacteria; HNP3 has lower activity than HNP1 and HNP2 for four out of five strains (Daher *et al.*, 1986). HNP1 was also found to have activity against a number of other enveloped viruses but was not active against the non-enveloped *echovirus* and *reovirus* (Daher *et al.*, 1986). HNP1 and HNP2 were also found to be active against the fungi *C.albicans* (Wilde *et al.*, 1989). HNP4 was shown to kill *E.coli*, *Streptococcus faecalis* and *C.albicans*. Compared to a mixture of the other human defensins, HNP4 was a hundred-fold more active against *E.coli* and four-fold more active against *S.faecalis* (Wilde *et al.*, 1989). Similar experiments have demonstrated antimicrobial activities for rabbit and rat defensins (Lehrer *et al.*, 1989). Activities of defensin molecules when assayed *in vitro* have demonstrated up to a 1000-fold variation in killing efficiency of various species of bacteria by different defensins. Variations in the potencies of these molecules may depend on the assay conditions as well as the target organisms. In *in vitro* assays, the level of defensin molecules is very low in comparison to that which may be achieved in the phagosomes, and *in vivo* defensins do not act in isolation but in concert with other antimicrobial defences which may help potentiate defensin activity.

The mechanism of defensin activity is unknown, however binding to a lipid bilayer is a prerequisite. A membrane potential is required as cells can be destroyed only when metabolically active, and can be protected by membrane depolarising agents such as carbonylcyanide M chlorophylhydrazone and 2,4-dinitrophenol (Kagan *et al.*, 1990). This result is consistent with the observation that defensins form voltage-dependent channels in lipid bilayers, a process which appears to involve an aggregation of 2-4 molecules. The crystal structure of HNP1 and HNP3 have been determined. This molecule crystalises as a dimer and unlike other channel-forming proteins contains no α-helical regions. The structure suggests a number of possible mechanisms of action, but no data are as yet available to judge between them (Hill *et al.*, 1991; Stanfield *et al.*, 1988).

1.2 (ii) Other activities of defensins

Other than the destruction of micro-organisms during phagocytosis, defensin molecules have a number of other activities. They may play a role in cytotoxicity at the site of infection and/or inflammation as they may be released at active concentrations into the extra-cellular environment (Ganz 1987). These molecules also display strong chemotactic activity for monocytes *in vitro*, and hence may be involved in the recruitment of monocytes to the site of infection and/or inflammation (Territo *et al.*, 1989). Defensins have been reported to act as corticostatins i.e. agents that interfere with the effect of adrenal corticotropin hormone (ACTH) on adrenal cortical cells by binding specifically to the ACTH receptor (Qinzhang *et al.*, 1987). An *in vitro* assay has demonstrated that defensins non-competitively inhibit protein kinase C activity, but what role if any this plays *in vivo* is unclear (Charp *et al.*, 1988).

1.3 Leukaemia

Leukaemia can be defined as the uncontrolled proliferation or expansion of haemopoietic cells that do not retain the capacity to differentiate normally into mature end cells. This distinguishes leukaemia from other haematological disorders that do not display a full leukaemic phenotype - either myeloproliferative (characterised by growth expansion) or myelodysplastic syndromes (which show abnormal differentiation). Both, however, can progress to acute leukaemia.

1.3 (i) Maturation Arrest

Most evidence supports the theory of leukaemia being monoclonal in origin, with the original transforming event taking place within the stem cell or immature progenitor compartments. For example chronic myeloid leukaemia (CML) is a

disease of the pluripotent stem cell, this disease is characterised in the chronic phase by an expansion in the myeloid compartment. The Philadelphia chromosome (a reciprocal translocation between chromosomes 9 and 22) characteristic of CML is found in all the haemopoietic lineages, but not in fibroblasts or bone marrow stromal cells indicating an origin of this lesion in the haemopoietic stem cell.

Comparison of the phenotypes of leukaemic and normal haemopoietic cells has revealed that the leukaemic cells have a phenotype similar to a precursor cell in normal haemopoiesis Greaves *et al.*, 1981; Foon and Todd 1986). This precursor under normal circumstances is restricted to the bone marrow, present in low numbers and is often transient in nature. In the leukaemic cell the processes of proliferation and differentiation have been uncoupled, leaving the cells 'maturation arrested' at a stage similar to that occurring during normal haematopoesis. It is this loss of the ability to differentiate terminally together with the capacity for continued proliferation that produces the leukaemic phenotype. This uncoupling of proliferation and differentiation may not be absolute, but may vary in stringency. The greater the uncoupling the less mature the phenotype of the transformed clone (Greaves *et al.*, 1982; Sachs 1982). If a relatively small shift in the balance between proliferation and differentiation occurs in the stem cell compartment, this may result in a gradual clonal expansion allowing cells to differentiate almost terminally. This pattern is seen in CML during the chronic phase, which is characterised by an expansion in the myeloid compartment with the presence of relatively mature cells in the peripheral blood. A more stringent uncoupling would explain the presence of leukaemic blast cells in the blood of acute leukaemia patients and during the blast crisis of CML. What are the candidate genes responsible for the phenomena of maturation arrest ? Possible targets are genes which are involved in the control of proliferation and differentiation.

1.3 (ii) Disorders of growth and differentiation

In acute leukaemia there is an increase in the number of circulating white blood cells and a defect in their normal maturation. The existence of myelodysplastic and myeloproliferative disorders suggests that these processes may be separable and full leukaemic transformation requires defects in both differentiation and growth control. Possible molecular mechanisms of leukaemogenesis are discussed in sections 1.3 (iii-iv). These are separated into defects in growth promoting genes and differentiation genes. However it should be remembered that in many systems, the processes of terminal differentiation and growth inhibition are normally stringently coupled.

1.3 (iii) Growth factors and leukaemia

Obvious candidates for oncogenes involved in leukaemogenesis are the haemopoietic growth factors and their cognate receptors. Reconstitution of mouse bone marrow and transgenic mice have been used assess the effect of constitutive expression of GM-CSF and IL-3. These generate a myeloproliferative disorders but not full leukaemia (Chang *et al.*, 1989; Johnson *et al.*, 1988; Wong *et al.*, 1989). Obviously another oncogenic change is necessary to generate full leukaemia. As well as over-expression of the growth factor itself, a number of other changes can result in growth factor independence. These include constitutive activation of the receptor or activation of a tyrosine kinase oncogene such as *abl*. CML is characterised at the molecular level by the generation of a fusion between the *bcr* and the *abl* gene. Introduction of the *bcr-abl* oncogene into haemopoietic cells renders them growth factor independent and tumourigenic in mice. The property of rendering cells growth factor independent is common to growth factor/tyrosine kinase oncogenes. However this growth factor independence does not always correlate with tumourgenicity *in vivo* and many tumourigenic myeloid cell lines

remain dependent on exogenous growth factors for growth *in vitro*. Clearly as growth factor over-expression alone is not sufficient to confer the complete leukaemic phenotype, is there evidence for an involvement in leukaemia *in vivo* ?

The first proto-oncogene identified as a haemopoietic growth factor receptor was c-*fms*, the cellular homologue of the v-*fms* - the transforming gene of the Feline Sarcoma Virus. V-*fms* was transduced from the cellular M-CSF receptor gene (Sherr *et al.*, 1985). The M-CSF receptor is active only in the presence of M-CSF. The viral form has constitutive tyrosine kinase activity, and reconstitution of mouse bone marrow with bone marrow infected with v-*fms* containing retrovirus results in malignancies of multiple haemopoietic lineages (Ridge *et al.*, 1990). Analysis of the mutations necessary for c-*fms* to be converted to an oncogenic form allowed the analysis of leukaemias for activated c-*fms*. This revealed that transforming mutations in c-*fms* were found in approximately 10% of AML and MDS patients tested (Ridge *et al.*, 1990)

IL-3 has also been implicated in the development of a sub-type of acute preB-cell leukaemia with a characteristic t(5:14) translocation. In two patients this was found to deregulate IL-3 expression by bringing the IL-3 promoter in close proximity to the immunoglobulin heavy chain gene (Meeker *et al.*, 1990).

1.3 (iv) Differentiation genes and leukaemia

Characterisation of retroviral insertion sites and cloning of chromosomal translocation break points in leukaemia has identified a number of genes which are strong candidates for playing a role in leukaemogenesis. A large number of these genes have similarity to genes encoding proteins of known transcription factor families, whose members are expressed in a developmental and differentiation-specific manner. These translocations often result in the abnormal expression, or alter the transcriptional properties of these genes by the generation of chimeric or truncated genes. This suggests that they may play a causal role in the process of

maturation arrest by interfering with normal differentiation.

An instructive and well characterised example is the translocation t(15:17) found in all patients with a sub-type of acute myeloid leukaemia, acute promyelocytic leukaemia. Cloning of the translocation site (de The *et al.*, 1991) defined the gene on chromosome 15 as *myl* or PML, whose sequence suggests it may encode a protein which forms a zinc finger structure and bind either DNA or RNA. The gene disrupted on chromosome 15 is the retinoic acid receptor α (RARα) gene. This translocation has the effect of generating a fusion gene encoding a protein containing the C-terminus of the retinoic acid receptor α (containing the DNA and ligand binding domains) and the N-terminus of the product of the PML gene. This leukaemia can be successfully treated with all *trans*-retinoic acid, presumably by overcoming the block to differentiation (which is observed when blast cells from these patients are treated *in vitro*, Breitman *et al.*, 1981). However, much higher than physiological levels are necessary to induce differentiation and remission in these patients. As the leukaemic cells still retain a normal copy of both RARα and the PML genes, the differentiation block must act in a dominant negative fashion.

How the PML-RAR fusion protein blocks differentiation is unclear, however it has the ability to activate transcription of gene expression via the retinoic acid receptor binding site (Pandolf *et al.*, 1991; de The *et al.*, 1991; Kastner *et al.*, 1992). This trans-activation is variable depending on the cells used and the response element itself. The finding by Pandolfi *et al.*, (1991) that the gene had dual activity, viz ligand independent repression and ligand dependent activation, would explain the differentiation block and the response to retinoic acid, although a number of other mechanisms are possible and further analysis will be instructive. Interestingly, transfection of a construct containing this fusion gene into the monocytic cell line U937 blocked differentiation of this cell line and reduced apoptotic death in the presence of low serum (Grignani *et al.*, 1993).

A similar example is provided by the thyroid hormone receptor family. The

acute erythroblastosis virus (AEV) induces rapid erythroleukaemia in chickens. This virus contains the oncogene *v-erb*A, the cellular homologue *c-erb*A encodes the thyroid hormone receptor (Sap *et al.*, 1986; Weinberger *et al.*, 1986). The thyroid hormone receptor binds DNA constitutively but activates transcription only in the presence of ligand. The viral form however binds DNA but fails to activate transcription even in the presence of high levels of ligand. This dominant repressor effect is believed to block differentiation (Zenke *et al.*, 1990; Damm *et al.*, 1993).

The AEV also contains a second oncogene, *v-erb*B, the viral counterpart of the epidermal growth factor receptor. Mutant viruses lacking *v-erb*B activity show no transforming activity, but mutant viruses defective in the *v-erb*A gene can transform erythroblasts. These transformed cells spontaneously differentiate at a high level underlining the co-operation of these two oncogenes, with *v-erb*B exerting its effects on growth and *v-erb*A in blocking differentiation.

The myelodysplastic syndromes may be a case where a lesion has occurred only in a gene(s) affecting normal differentiation without generating a proliferative advantage. The peripheral blood of these patients is characterised by low levels of leukocytes, however the marrow has normal or slightly elevated levels of precursor cells. The molecular mechanisms generating these conditions are unknown although oncogene abnormalities such as activating mutations of ras and p53 have been reported (Bos 1989). Treatment of these patients with CSFs has successfully increased the number of circulating white blood cells, but interestingly, examination of the bone marrow show that this treatment produces some of the features of acute leukaemia in a number of patients. This phenotype is lost when the CSF is withdrawn, suggesting that to generate acute leukaemia a change which can functionally substitute for the presence of exogenous growth factor is necessary in these cells (Ganser *et al.*, 1990).

A recent report identified a fusion protein generated by the t(3:21) found in some therapy-related myelodysplastic syndromes, acute myeloid leukaemia and the blast crisis of CML. This translocation fuses the AML1 gene (which has homology

to the *Drosophila* segmentation transcription factor *runt*) to the gene encoding the ribosomal protein L22. The mechanism of action of the fusion protein expressed in the cells is unknown. However, it may act in a dominant negative manner to block the normal activity of the AML1 transcription factor as the truncated AML1 gene generated by the translocation is postulated not to contain the the sequences encoding the transactivation domain. As this translocation is found in both acute myeloid leukaemia and in the blast crisis but not in the chronic phase of CML, studies of the mechanism of action of this protein may shed light on the process of maturation arrest.

Other nuclear acting oncogenes can also block differentiation, these include EVI-1 and Myb. These fail to confer growth factor independence onto factor-dependent cell lines and do not increase their proliferation rates (reviewed in Ihle and Askew 1986).

1.3 (v) Apoptosis and leukaemia

The *bcl*-2 gene is associated with the t(14:18) chromosomal translocation present in 85% of follicular lymphomas (Fukuhara *et al.*, 1979). The sequence of the gene suggests that *bcl*-2 may encode a GTP-binding protein. This rearrangement joins the *bcl*-2 gene at its 3' untranslated region to an immunoglobulin heavy chain J segment, resulting in deregulated expression of *bcl*-2 (Tsujimoto *et al.*, 1985; Bakhshi *et al.*, 1985; Cleary and Sklar 1985)

Bcl-2 appears to act in a novel fashion in that it can promote cell survival without stimulating proliferation in growth factor dependent cell lines deprived of cytokines (Nunez *et al.*, 1990). Transgenic mice with constructs similar to the fusion generated by the t(14:18) translocation recreate the pathology seen in follicular lymphoma (McDonnell *et al.*, 1989). These transgenic animals develop lymph node and splenic enlargement as a result of a polyclonal expansion of mature B cells resting in G0. As in the human disease 10% of these animals go on to

develop clonal large cell lymphomas. Around half of these lymphomas have acquired translocations activating the c-*myc* gene. (McDonnell and Korsmeyer 1991). Transgenic mice containing c-*myc* and *bcl*-2 under the control of a B-cell specific promoter die at 5-6 weeks of age as a result of malignant lymphoma (Strasser *et al.*, 1990). The ability of *bcl*-2 to act as an oncogene by preventing cell death is a novel mechanism. The extended lifespan of this population of cells will give it an increased chance of acquiring other mutations necessary for full transformation. The cooperativity between *c-myc* and *bcl*-2 is interesting in the light of the role of c-Myc in mediating apoptosis.

The ability of c-Myc to mediate apoptosis has been demonstrated in IL-3 dependent cell lines. Constitutive expression of *c-myc* in these cells when deprived of IL-3 drives cells into the cell cycle and increases apoptotic cell death (Askew *et al.*, 1991). Similarly activation of Myc in quiescent fibroblasts results in apoptosis. However expression of *bcl*-2 specifically abrogates apoptosis without affecting the mitogenic function of c-Myc (Fanidi *et al.*, 1992). It has been suggested that two signals are necessary: c-Myc mediates a proliferative and an apoptotic signal and a second signal is necessary to suppress the apoptosis. This suggests that activation of Myc in non-transformed cells would lead to apoptosis and activation of Bcl-2 or a tyrosine kinase oncogene would allow this apoptosis to be suppressed.

1.4 Lineage commitment

1.4 (i) Lineage infidelity

The classical representation of haemopoiesis depicts a hierarchical structure with cells increasingly restricted in lineage 'choices' as they mature, eventually becoming committed to a single lineage. This scheme of haemopoiesis would predict that markers normally restricted to different lineages would not be co-expressed on a single cell. However analysis of leukaemic phenotypes has revealed

a number of examples of this co-expression of 'lineage specific' markers. McCulloch has argued that this 'lineage infidelity' is the result of abnormal gene expression as a consequence of leukaemic transformation (McCulloch 1987). An alternative explanation is that these leukaemic cells are maturation arrested at an early stage of differentiation prior to lineage commitment. If the latter explanation is correct then what does this co-expression of normally lineage restricted markers suggest about the process of lineage commitment in normal haemopoiesis?

A number of these documented cases of lineage infidelity can be discounted on technical grounds (Greaves 1986), however some are genuine and merit further analysis. For example, expression of terminal deoxynucleotidyl transferase (which is normally restricted to immature lymphocytes) has been found in leukaemic myeloblasts (Bollum 1982; Jani *et al.*, 1983) and T-cell receptor gene rearrangements have been detected in inappropriate lymphoid or myeloid leukaemic cells (Furley *et al.*, 1986).

Greaves has suggested that rather than aberrant gene expression, the co-expression of markers of different lineages occurs in normal haemopoiesis during a period of 'lineage promiscuity' before a cell becomes committed to a particular lineage. If a leukaemia arose in a pre-commitment phase of haemopoiesis and was subject to a relatively stringent maturation arrest, then this would present itself as lineage infidelity. Limited or selective promiscuity could occur with respect to genes that are the first to be expressed in a lineage and whose activity is perhaps functionally involved in commitment. Candidates for these genes would include the haemopoietic receptors which could serve to 'advertise' lineage options and the genes necessary for successful recombination of T-cell receptor / immunoglobulin genes, where successful recombination may commit a precursor to a particular lineage.

Does lineage infidelity follow this prediction in that early markers of a lineage are more likely to be involved in lineage infidelity? This prediction is borne out by the analysis of rearrangements of the immunoglobulin genes. A large number

of reports have identified recombination of the heavy chain genes in non pre-B cell leukaemias, however to date no reports of recombination of the light chain genes have been reported in these leukaemias (Korsmeyer *et al.*, 1981; Rovigatti *et al.*, 1984; Ha *et al.*, 1986). This is instructive as rearrangement of the heavy chain genes occurs earlier in pre B-cell development than recombination of the light chain genes (Korsmeyer *et al.*, 1981).

An early haemopoietic marker is terminal deoxynucleotidyl transferase (TdT). TdT is expressed in leukaemia and normal pre-B and pre-T cells but not in mature lymphocytes and is involved in the generation of diversity of T-cell receptor and immunoglobulin genes (Siu *et al.*, 1984). Despite the lineage-restricted nature of this marker, it is found in 5-10% of acute myeloid leukaemias (Jani *et al.*, 1983; Coleman and Hutton 1982). In a number of cases it has been unambiguously demonstrated that TdT and myeloperoxidase (a myeloid marker) coexist in the same cell (Lanham *et al.*, 1984). Interestingly, although TdT has been identified in the blast crisis of CML, no TdT has been identified in the chronic phase granulocytes, despite the origin of this leukaemia in the pluripotent stem cell compartment (Coleman and Hutton, 1982; Bradstock *et al.*, 1981 ; Hoffbrand *et al.*, 1977). This suggests that the progressive differentiation of these leukaemic cells allows these markers to be switched off after irreversible lineage commitment.

1.4 (ii) Models of lineage commitment

Two basic models have been proposed to explain the renewal, differentiation commitment in stem cells. In perhaps the favoured model, lineage's are restricted in a stochastic or deterministic fashion, forming pluri, oligo, bi-potent and ultimately committed cells. This process is proposed to occur at the genetic level and is independent of the influence of external factors (Till *et al.*, 1978; Ogawa *et al.*, 1983). The alternative model proposes that progenitor cells are intrinsically flexible and external factors such as growth factors, stromal elements

or the haemopoietic micro-environment determine the pathway of differentiation (Trentin 1971; Dexter 1982).

The lineage promiscuity model explains lineage infidelity by the co-expression of markers which may be functionally involved in the choice of lineage. This is clearly a deterministic model of lineage commitment. Obvious candidates for lineage promiscuity are the haemopoietic growth factor receptors, which could be co-expressed on progenitor cells and serve to 'advertise' available lineage choices, which would be determined by the relative levels of haemopoietic growth factors. Many of the haemopoietic growth factors and their cognate receptors have been cloned and characterised. This has allowed experimental analysis to determine if these factors are involved in the process of lineage determination.

Lineage switching of 'committed' bone marrow cells has been observed on the introduction and expression of certain growth factors, receptors or oncogenes. Two examples demonstrate that lymphoid cells can differentiate into macrophages given the appropriate signals. Pre-B cells from transgenic mice containing a c-*myc* gene under the control of a B-cell specific promoter become macrophage like after the introduction of v-*raf* (Klinken *et al.*, 1988). Similarly, infection of normal bone marrow with a retrovirus expressing both v-*myc* and v-*raf* generates both pre-B and macrophage clones derived from the same progenitor cell (Principato *et al.*, 1990). As v-*raf* activates c-*fms* (M-CSF receptor) it is possible that this receptor is responsible for this lineage switch. This possibility is borne out by the introduction of c-*fms* into the murine bone marrow culture. These cultures switch from lymphoid to macrophage in the presence of M-CSF, but this lineage switch is over-ridden by the addition of IL-7 to the culture (Borzillo *et al.*, 1990).

A similar deterministic function for colony stimulating factors (CSF) in lineage commitment has been suggested using the mulipotent murine line FDC-P mix (Spooncer *et al.*, 1986). These cells depend on exogenous IL-3 for survival and proliferation, but can be induced to differentiate by reducing IL-3 levels and adding an appropriate growth factor. Single FDC-P mix cells infected with

retroviral vectors expressing GM-CSF are induced to differentiate to granulocytes and macrophage, suggesting GM-CSF induces the differentiation by an instructive mechanism (Just *et al.*, 1991).

A difficulty with many of these studies is that they are carried out in the presence of haemopoietic growth factors. These factors act as survival and mitogenic factors in addition to any role they may have in lineage determination. Uncoupling of these processes is difficult as the cells require these factors to remain viable, and much evidence suggests that rather than acting by determining lineage choice, these expand a compartment of committed cells expressing the appropriate receptor. Evidence that the differentiation pathway is determined intrinsically rather than via the receptor is that non-haemopoietic receptors can induce proliferation and differentiation when expressed in haemopoietic cells in presence of the appropriate ligand. This differentiation is these experiments is clearly cell-specific rather than determined by the receptors (Pierce *et al.*, 1988; Wang *et al.*, 1989). Similarly alterations in cell physiology indicating different signalling pathways for different haemopoietic receptors has not been observed, suggesting these receptors mediate common signals. These results suggest that rather than determining lineage commitment these haemopoietic growth factors act via expanding a compartment of committed cells via a proliferation signal which is coupled with a signal to differentiate along a lineage which has been predetermined.

An uncoupling of the survival signal from the mitogenic signalling of the CSFs has been achieved by generating cell FDC-P cell lines which constitutively express *bcl*-2. Expression of this gene has been demonstrated to delay apoptosis in factor dependent cell lines on withdrawal of growth factor (Nunez *et al.*, 1990). When IL-3 was withdrawn from these cells lines, they showed continued survival in both serum-containing and serum-free conditions. This survival was accompanied by multi-lineage differentiation, which was not the result of autocrine production of haemopoietic growth factors. These results suggest that the lineage determination can be intrinsically determined without the influence of external

factors (Fairbairn *et al.*, 1993). Although these results suggest that external factors are not obligatory for lineage determination, this does not mean that they are unable to influence this lineage choice.

1.5 Human myeloid cell lines

Much of the work on myeloid differentiation has been undertaken using cell lines established from patients with either acute myeloid leukaemia or in blast crisis of CML. These cell lines are maturation arrested at different stages of differentiation and many can be induced to differentiate with the addition of a variety of physiological and non-physiological agents (reviewed in Hozumi 1985). These lines have been used as model of haematopoeitic differentiation, however they share abnormalities (both cytogenic and molecular) characteristic of the leukaemia from which they were derived as well as other changes which are presumably necessary for growth *in vitro*. The genetic changes necessary for leukaemic cells to become immortalised are unknown, making the generation of leukaemic cell lines a difficult and essentially fortuitous process (Koeffler 1984).

A large number of cell lines have been established which have a phenotype representative of normal haemopoietic counterparts. For example KG1 was established from a patient with erythroleukaemia (Koeffler and Golde 1978). This cell line is myeloblastic in nature and can be induced to differentiate towards macrophage by a number of agents. The K562 cell line was established from a patient in blast crisis of CML (Lozzio and Lozzio 1975). These cells are arrested at an early myeloblast / erythroblast stage, and can differentiate towards erythrocytes when cultured with hemin or butyrate (Miller *et al.*, 1984). Many other cell lines have been established representative of different stages of haemopoietic differentiation e.g. megakaryoblastic CMK (Sato *et al.*, 1987), monoblastic U937 and THP-1 (Sundstrom and Nilsson 1976; Tsuchiya *et al.*, 1980), myelomonoblastic ML1, ML2 and ML3 (Takeda and Minowada 1982) and

eosinophil precursor like cell lines EoL-1 and EoL-3 (Saito *et al.*, 1985). Of particular importance to this study is the promyelocytic cell line HL60, and this is discussed in more detail in section 1.5 (i).

1.5 (i) HL60 as a model system for human myelopoiesis

This cell line was derived from a patient with acute non-lymphocytic leukaemia. It is an immortal, factor-independent cell line with distinct myeloid characteristics (Collins *et al.*, 1978; Gallagher *et al.*, 1979). The cell line is composed predominantly of cells which have the morphological and biochemical characteristics of promyelocytes. Most cultures particularly those which are passage 60 or less show spontaneous differentiation with around 5% of cells showing features of more mature myeloid cells such as myelocytes, metamyelocytes and PMN (Collins *et al.*, 1977). The ability of this cell line to differentiate in response to a range of inducing agents down a number of different pathways has made this cell line a particularly useful model for human myeloid differentiation. The ability of HL60 cells to proliferate continuously *in vitro* is in contrast to the properties of fresh leukaemia cells, which undergo a limited number of cell divisions before growth arrest and cell death. What genetic changes the HL60 cell line has undergone to become immortalised are unknown, although autocrine factors have been purified from the HL60 conditioned medium (Brennan *et al.*, 1981; Perkins *et al.*, 1984).

HL60 cells show many karyotypical abnormalities, a number of which have been described including monosomy, polysomy and a number of chromosomal translocations (Wolman *et al.*, 1985; Donti *et al.*, 1988). A number of specific lesions have been identified, which may have played a role in generating the original leukaemic phenotype and/or the immortalisation *in vitro*. The HL60 cell line lacks a functional p53 gene: the cells are monosomic for chromosome 17 (Gallagher *et al.*, 1977) and the other allele contains a deletion (Wolf and Rotter

1985). The N-*ras* gene contains an activating codon 61 mutation (Bos *et al.*, 1984) and this is responsible for the transforming activity of HL60 DNA in an NIH3T3 transformation assay (Murray *et al.*, 1983). Other acute myeloid leukaemias exhibit N-*ras* mutations (Bos *et al.*, 1985; Needleman *et al.*, 1986), but the functional significance of the preferential activation of mutations in N-*ras* rather than other members of the *ras* family in myeloid leukaemias is unknown.

The c-*myc* gene is amplified in the HL60 cell line as well as in the primary leukaemic cells (Collins and Groudine *et al.*, 1982). The loss of expression of this gene is a rapid event following induced differentiation of HL60. The use of antisense oligonucleotides to c-*myc* results in the partial differentiation of HL60 cultures, suggesting that the amplification and consequent high levels of c-Myc blocks differentiation in this cell line (Yokoyama and Imamoto 1987; Holt *et al.*, 1988; Wickstrom *et al.*, 1988).

1.5 (ii) HL60 differentiation

The HL60 cell is morphologically myeloblastic/promyelocytic in nature, with a characteristic large rounded nucleus containing 2-4 distinct nucleoli, and a basophilic cytoplasm with azurophilic granules. The cell line also carries a number of markers chararcteristic of promyelocytes, including myeloperoxidase, acid phosphatase and receptors for lactoferrin, insulin and complement (reviewed in Collins 1987). The well-characterised nature of this cell line along with its ability to differentiate to either monocytes/macrophage like cells or granulocytes and ease of handling makes it a useful model of human myelopoiesis.

A variety of compounds induce granulocytic differentiation including dimethylsulphoxide and retinoic acid. This is accompanied by a progressive decrease in cell size and condensation of the nuclear material with the appearance of kidney-shaped or lobed nuclei characteristic of banded and segmented neutrophils. The ratio of nucleus to cytoplasm decreases and the cytoplasm becomes more

diffuse. A large number of biochemical changes can detected, including decreased myeloperoxidase activity and the appearance in the culture of cells capable of reducing nitroblue tetrazolium (a marker of mature neutrophils). A number of features of this differentiation program illustrate that it is incomplete or defective. For example most cultures induced to differentiate to granulocyte-like cells consist primarily of metamyelocytes and banded neutrophils rather than fully differentiated multi-lobulated PMN. In addition, these differentiated cells lack lactoferrin suggesting that they are deficient in secondary granules (Newburgher *et al.*, 1979; Olsson and Olofsson 1981).

In contrast treatment of HL60 cells with TPA (which induces monocytic differentiation) leads to the cells clumping and adhering to the tissue culture surface within 24hrs. These cells spread out and acquire a spindle like morphology with prominent pseudopodia. There is a decrease in myeloperoxidase activity and the appearance of non-specific esterase activity (a monocyte/macrophage marker). These differentiated cells also show functional changes with both HL60 granulocytes and monocytes/macrophage capable of phagocytosis (Collins *et al.*, 1978; Polansky *et al.*, 1985).

Chapter 2: The regulation of transcription

2.1 The role of chromatin in eukaryotic gene control

Although many discussions on the control of transcriptional initiation centre on the binding of transcription factors to their appropriate sites as assayed by DNA-protein interactions *in vitro*, clearly the *in vivo* situation is more complex as chromatin, and not naked DNA, is the template for transcription.

The basic unit of chromatin is the nucleosomal core or histone octamer around which 166bp of DNA are wound approximately 1.8 times to form the 'beads on a string' 10nm fiber. This fiber undergoes further condensation mediated by the histone H1 binding in the nucleosomal linker to form the 30nm fiber. How this fiber is folded into higher order condensed structures (such as heterochromatin or fully condensed chromosomes) or the molecular organisation of such structures is not well understood.

Transcriptionally active chromatin in contrast to the bulk of chromatin is generally more unwound than that of transcriptionally silent chromatin. Classically this has been assayed by sensitivity to nuclease digestion (Wu *et al.*, 1979a and b). This approach has revealed that the chromatin surrounding transcriptionally active (or inducible genes) is more sensitive to nuclease digestion (usually over many kilobases) than that of bulk chromatin. As well as increased sensitivity over a large domain, the chromatin surrounding active genes is normally characterised by the presence of a number of small sites (50-400bp) which show extreme sensitivity to nucleases. These are termed DNAse hypersensitive sites (DHS). These appear to be gaps in the nucleosomal array, however whether the nucleosome is totally displaced or just less stringently bound to DNA is not clear. Evidence of the latter is that cross-linking studies have demonstrated the DHS generated by the glucocorticoid receptor binding at the mouse mammary tumour virus (MMTV) LTR can still be cross-linked to nucleosomal histones (Bresnick *et al.*, 1992).

These DHS have been demonstrated to be bound by non-nucleosomal proteins both *in vitro* and *in vivo* by a number of studies, and are necessary for the transcriptional control of a number of genes (for review see Gross and Garrard 1988). That areas of DNA important for transcriptional control (i.e. the binding of transcription factors) are often DHS in active genes suggests that nucleosomes may play a role as general repressor of transcription. However it could be argued that the DHS are of no functional importance and are simply displaced as a result of transcription factor binding. That nucleosomes act as general repressors has been investigated both biochemically and genetically, and the evidence is discussed in the following section 2.1 (i).

2.1 (i) Nucleosomes as general repressors of transcription

The study of several genes has demonstrated that nucleosome positioning can have important functional consequences for gene control. The yeast acid phosphatase PHO5 gene is inducible in low phosphate media and has a number of functionally important upstream activating sequences (UAS). The positions of the nucleosomes over the promoter and upstream have been precisely mapped (Almer and Horz 1986). One of these UAS is within a DHS present in both inducing and non-inducing conditions. In low phosphate media the product of the PHO4 gene binds to this UAS which leads to the displacement of four nucleosomes revealing a PHO4 binding site and the TATA box (Almer *et al.*, 1986; Almer and Horz 1986). Another transcription factor PHO2 can also bind to a site between the two PHO4 binding sites; however, *in vivo* footprinting revealed that this protein could only recognise this site in nucleosomal but not non-nucleosomal DNA. Hence PHO2 may facilitate the displacement of this nucleosome, as over-expression of PHO4 at high levels in high phosphate media leads to the loss of this nucleosome only in the presence of PHO2. However over-expression of PHO2 has no effect on chromatin structure (Fascher 1990).

Another example of nucleosome positioning repressing transcription by preventing access of transcription factors to their control sequences is the glucocorticoid mediated transcription from the MMTV promoter. In this case the glucocorticoid receptor (GR) binds its recognition sequence within a nucleosome displacing it and allowing access to another transcription factor binding site (which is bound by NF1) site and the TATA box (Richard-foy and Hager 1987; Archer *et al* 1992). The NF1 protein is unable to bind to nucleosomal DNA despite being able to bind to this sequence in naked DNA with high affinity. Similarly the enhancer of the rat tyrosine aminotransferase gene can be bound and activated by the GR when bound by nucleosomes (Riek *et al.*, 1991). This example illustrates that some transcription factors can recognise sites within a nucleosomal array and that others can only bind to DNA when exposed in a DHS.

These examples suggest that setting up a positioned nucleosomal array in which some gaps are present (such as in the PHO5 gene) may have important regulatory consequences. However what determines the precise positioning of nucleosomes is an important question. Nuclesomes show some sequence specificity *in vitro*, and artificial sequences that have 10bp periodicity which are predicted to bend easily show 100-fold more specificity for nucleosomes than bulk DNA (Shader and Crother 1989). However this level of affinity is unlikely to account for the positioning affects seen *in vivo*. Another explanation proposed is that higher order folding of chromatin modulates the placing of nucleosomes (Thoma 1988). Many DHS have been demonstrated to be bound by non-histone proteins suggesting that DNA binding proteins may bind certain sequences and act as an anchor around which nucleosomes are placed. These proteins could bind to the DNA during replication or directly to the nuclesomal array and displace nucleosomes as is the case with the GR.

The above examples suggest that the precise positioning of nucleosomes has important regulatory functions. The use of genetic approaches have conclusively demonstrated that the nucleosomes themselves are negative regulators

of transcription. The construction of yeast strains in which expression of the histone H4 gene has been brought under the control of an inducible promoter has demonstrated that altering histone stoichiometry can alone induce the expression of a number of genes including PHO5, CUP1 and HIS3 (Han *et al.*, 1988; Durin *et al.*, 1992).

Biochemical evidence that nucleosomes can repress gene expression comes from the reconstitution of transcription *in vitro*. A number of studies have demonstrated that the preassembly of nucleosomes onto transcriptional templates drastically inhibits transcriptional initiation (reviewed by Workman *et al.*, 1992). Addition of the basal transcription factor TFIID prior to nucleosomal assembly relieves this repression (Workman and Roeder 1987). Using the adenovirus major late (Ad ML) promoter as a model it was demonstrated that inclusion of USF transcription factor as well as TFIID further increased transcription *in vitro*. It was suggested that this transcription factor was acting via stabilising/facilating TFIID binding as both factors had to be present prior to nucleosome assembly to function. In contrast to these results, in experiments with GAL4 derivatives, addition of TFIID after nucleosome assembly was still effective in stimulating transcriptional initiation. The transactivation domain was necessary for this activity (Workman *et al.*, 1991). These results suggest that there may be two different modes of transcriptional activation, one may be to facilitate/stabilise preiniatiation complex formation without a direct role in the destabilising of nucleosomes. Strong evidence for this mode of transcriptional activation is that many transcription factors (albeit with much reduced efficiencies) can function *in vitro* on naked DNA templates (Chasman *et al.*, 1989; Carey *et al.*, 1990). The other mode of transcriptional activation is the destabilisation of nucleosome-DNA interactions allowing binding of basal and/or other transcriptional activators which can recognise nucleosomal DNA (cf. activation of the MMTV promoter). Both of these functions are likely to be important; for example, with naked DNA templates Sp1 and GAL4-VP16 stimulated *in vitro* transcription 30-fold and 8-fold respectively. However with

chromatin templates containing histone H1 transcriptional activation mediated by these factors was 90 and 200 fold higher. This is the result of both true activation and the relief of chromatin mediated repression (Laybourn and Kadonaga 1991).

Genetic analysis of the regulation of specific genes has identified mutants which have a global effect on the regulation of a number of genes. These mutations must be in genes encoding the general transcription machinery or be involved in modulating chromatin structure. Studies on the SUC2 gene in yeast (an invertase necessary for sucrose fermentation) revealed three mutants which also affected the expression a number of other genes. The mutants *swi1*, *swi2* and *swi3* are involved in the regulation of SUC2 expression, but do not appear to exert their effects through UAS of this gene (Peterson and Herskowitz 1992). Isolation of suppressor mutations suggested that these genes are involved in the modulation of chromatin structure. With one suppressor mutation in a gene with homology to HMG-1 (implicated in mediating chromatin structure, Kruger and Herskowitz 1991), and the others affecting histone stoichiometry (Winston and Carlson *et al.*, 1992).

2.1 (ii) Characteristics of active versus silent chromatin

Active chromatin as well as being in a more decondensed state (or DNAse1 sensitive) than bulk chromatin, it also has a number of other features; it is depleted of (or contains more loosely bound) histone H1, the DNA is undermethylated and contains higher levels of acetylated histones.

Histone H1

This histone binds the internucleosomal linker and is necessary for the formation of higher order chromatin structure. Purification of transcriptionally active chromatin from *Xenopus* oocytes has been found to be depleted of histone H1 (Wolffe 1990; Garrard 1989). This is consistent with the ability of histone H1 to inhibit *in vitro* transcription on reconstituted chromatin templates (Laybourne and

Kadonaga 1991). However, other studies have shown that both inactive and active chromosomal regions in chicken erythrocytes are associated with H1 and H5 (a tissue specific histone H1), but the internucleosomal linkers of the inactive genes are less sensitive to nuclease digestion suggesting an altered binding (Weintraub 1984). The role of histone H1 in organising DNA into higher order structures would suggest that there must be some decondensation to allow the transcriptional machinery access to the transcriptional control sequences.

Methylation

The correlation between transcriptional inactivity and methylation has been noted in many studies. The methylation patterns are clonally inherited and have been suggested to form the basis of a stable regulatory system (Riggs 1975). Although many studies have suggested a role for methylation in the repression of transcription most of the data has been correlative. This and the lack of appreciable methylation in either *Drosophila* or *Caenorhabdits elegans* genome (Urieli-Shovel 1982; Simpson 1986) cast doubt on the importance of methylation. However that methylation plays a crucial role in higher eukaryotes has been demonstrated by the generation of homozygous null mice for methlytransferase. These mice did not survive past mid-gestation and were stunted and delayed in development (Li *et al.*, 1992). Although clearly demonstrating the importance of methylation in higher eukaryotes these knockouts did not shed light on the role of methylation. Recent work has demonstrated that methylation plays a direct role in the repression of transcription. There appears to be two mechanisms for the repression of promoters by methylation, one of which is the methylation of transcription factor binding sites. A number of transcription factors fail to bind to their sequences when the latter are methylated (reviewed in Tate and Bird 1993). However, this cannot be the only mechanism of inhibition as the γ-globin promoter is repressed by methylation but mutagenesis studies demonstrated that the methylation does not have to be at any particular site (Murray and Grosveld 1987).

The second type of repression may be mediated by proteins which specifically bind methylated DNA, and a number of proteins with this property have been characterised. The methyl CpG binding protein MeCP1 can bind to sequences containing multiple symmetrically methylated CpGs. This protein can repress the transcription of methylated promoters *in vitro*. Embryonic stem cells have low levels of MeCP1, and methylation fails to inactivate promoters in these cells (Boyes and Bird 1991). Repression by this protein depends on both the density of methylation and the strength of the promoter. A study by Boyes and Bird (1992) demonstrated that repression of a weakly methylated promoter could be relieved by addition of an SV40 enhancer but not that of a heavily methylated promoter. A second protein MeCP2 has been cloned which can bind to a single symmetrically methylated CpG pair and can repress transcription in a similar manner. These proteins may act via direct interference or perhaps by mediating a change in chromatin structure.

Another protein was identified as binding to a methylated CpG in the vitellogenin gene which inhibited *in vitro* transcription (Jost *et al.*, 1991). Purification of this protein suggests that it is histone H1, although its binding properties and molecular weight are not the same as those previously noted (Jost *et al.*, 1991). Evidence has suggested histone H1 may have a preference for methylated DNA. This work demonstrated that methylated plasmid DNA incubated with histone H1 is less sensitive to nucleases than its unmethylated counterpart (Higurashi and Cole 1992). As methylated DNA is enriched in histone H1 *in vivo* , it may play a role in remodelling of chromatin (Ball *et al.*, 1983).

There appear to be two classes of promoter with respect to methylation: those which are constitutively non-methylated and CpG rich (CpG islands) and those which are CpG poor and methylated in most tissues, often corresponding to tissue-specific genes (Bird *et al.*, 1986). In cultured cell lines many CpG islands become methylated e.g. in NIH 3T3 and L cells, 50% of CpG islands tested were methylated. However, these CpG islands are not methylated *in vivo* even at

transcriptionally silent promoters, suggesting that non-essential genes can be switched off in tissue culture by this mechanism (Antequera *et al.*, 1990). An example is the CpG island at the *myoD* promoter which is methylated *in vitro* in 10T1/2 cells. Treatment of these cells with 5-azacytidine relieves this methylation-mediated repression and the cells differentiate to muscle cells. This is in contrast to the *in vivo* situation where this CpG island is unmethylated in all tissues (Jones *et al.*, 1990).

Histone acetylation

The amino terminal ends of the of the core histones all contain lysine residues which are reversibly methylated during the cell cycle. Treatment of cells with the deacetylating agent sodium butyrate leads to a partial unfolding of the interphase chromosomes (Annunziato *et al.*, 1988). This and the observation that active chromatin is selectively enriched for acetylated histones has suggested that acetylation may play a role in regulation of chromatin structure (Allegra *et al.*, 1987; Risdale *et al.*, 1987). That acetylation is not simply a consequence of transcription has been demonstrated in *Tetrahymena* using indirect immunofluorescence with antibodies for acetylated lysine residues which reveals that acetylation of histone H4 precedes transcription (Pfeffer *et al.*, 1989).

Genetic evidence has suggested that histone tails play a role in mediating transcription factor access to DNA. Mutation in the H4 tails can prevent the activation of some genes (Durrin et al., 1991) and the repression of others (Kayne *et al.*, 1988, Johnson *et al.*, 1990). Acetylation has also been implicated in the positioning of nucleosomes; the yeast repressor $\alpha 2$ positions nucleosomes when binding an operator in a minichromosome, however when the histone H4 tails are mutated, the location and/or stability of the nucleosomes is altered (Roth *et al.*, 1992).

2.2 Transcriptional control sequences

An important difference between prokaryotic and eukaryotic gene control is the existence of three RNA polymerases in eukaryotes. Each of these polymerases transcribes a specific subset of genes: RNA polymerase I transcribes the genes encoding the ribosomal RNAs 28S, 18S and 5.8S, RNA polymerase III transcribes the genes encoding tRNAs and small nuclear RNAs whereas RNA polymerase II transcribes the protein-coding genes and some small nuclear RNAs. The *cis*-acting sequences responsible for mediating the control of the class II genes are discussed below. These are simplistically classified into promoters and enhancers, the promoter is close to the site of transcriptional initiation, and enhancers are characterised by their ability to influence transcription up to many kilobases away from the promoter.

2.2 (i) Promoters: The TATA box and initiator elements

A characteristic of the promoters of many class II genes (those genes transcribed by RNA polymerase II) is the presence of a TATA box, which is usually centred around -28 with respect to the transcriptional start site. In fact, comparison of 168 class II promoters for sequence homology revealed that this was the only well conserved element, although this element itself appears to be absent from many class II genes. Mutational analysis have demonstrated the importance of this element in basal transcription (Grosveld *et al.*, 1982), controlling the specificity of transcriptional initiation, mediating the activity of some upstream activating sequences and determining the direction of transcription (O'Shea-Greenfield *et al.*, 1992a and b).

In higher eukaryotes the TATA box directs the formation of the preinitiation complex, in which RNA polymerase II is poised to begin transcription at a distance from the TATA box of 25 to 30 nucleotides (reviewed in Sawadogo and Sentenac

1990). The first step in this process is the binding of transcription factor II D (TFIID) to the TATA box, this is followed by the incorporation of TFIIB, TFIIF RNA polymerase II and TFIIE. As TFIID binds the TATA box in sequence specific manner, and as the preinitiaton complex forms in a directional manner (Sawadogo and Roeder 1985; Nakajima *et al.*, 1988; Buratowski *et al.*, 1989) it is conceptually easy to imagine how this sequence could determine the transcription start site by precisely positioning RNA polymerase in the preinitiation complex.

Many genes which lack a TATA box are characterised by multiple transcriptional start sites lending support to its role in determining the site of transcriptional initiation. However this is not the only element important in the determination of transcriptional initiation. Studies with a number of genes e.g. SV40 (Ayer and Dynan 1988), terminal deoxynucleotidyl transferase (Smale and Baltimore 1989) and dihydrofolate reductase (Mean and Farnham 1990) have identified elements which overlap the transcriptional start site and can influence its placement. These elements have been termed initiator elements or Inrs. The mechanism of action of these elements in unknown, however proteins which bind to these sequences have been identified (Seto *et al.*, 1991). These elements have been identified in both TATA containing and TATA-less promoters, and some workers have attempted to assess the relative contributions of the TATA box and Inr elements (O'Shea-Greenfield *et al.*, 1992). This study varied the position of a TATA box and the TdT Inr element and suggested that both could direct placement of the start site and act cooperatively together, however the TATA box appeared to be the dominant element in determining the start site during both basal and activated transcription.

Transcriptional initiation determination is more complex than the above description would suggest, with the TATA box as the primary determinant and the Inr elements playing a subsidiary role, as the TATA box does not always occur approximately 25-30 bp upstream of the CAP site. For example the Adenovirus IVa2 gene contains a functional TATA box downstream of the CAP site (Carcamo

et al 1990) and mutations in the promoter of the CYC-1 gene of *Saccharomyces cerevisiae* which contains three functional TATA boxes does not alter the start sites as would be predicted by the simple model discussed above (Han *et al.*, 1985). Another case where elements other than the TATA box may be important in determining the transcriptional start site and direction is where the TATA box is symmetrical, it may be that other factors such as chromatin structure may be important in this process *in vivo*.

Possibly all genes (including class I and III) require functional TATA binding protein (TBP) to allow transcriptional initiation to occur as this factor has been demonstrated to be necessary for a number of class II genes that lack an apparent TATA box as well as a number of class I and III genes (reviewed in White and Jackson 1992). It is not clear whether any class II genes lack a TATA box in the functional sense, as the SV40 ML promoter contains an element at -30 with no apparent homology to the TATA consensus but with similar properties. TBP can bind to this sequence, albeit with an affinity one sixth that of the Ad ML promoters TATA box. Mutational analysis of this element reveals that similarly to the TATA box; it can direct RNA polymerase II to initiate transcription approximately 30bp downstream of its location, inactivation leads to increased heterogeneity in the site of transcriptional initiation and point mutational analysis revealed that the affinity of TBP for this element correlated with the efficiency of transcription. All five other TATA-less promoters tested in this study bound the TBP at a site around -30, suggesting that many TATA-less promoters differ from TATA containing promoters only in the affinity of their -30 regions for binding of this basal transcription factor (Wiley *et al.*, 1992).

2.2 (ii) Transcriptional activating sequences

As well as these basal elements, a number of other *cis*-acting sequences have been identified in functional studies, which can mediate elevated levels of

transcription either constitutively, in a tissue-specific or developmental-specific manner or in response to a signal such as a cytokine or growth factor. These elements function by binding sequence-specific transcription factors which elevate or repress the activity of the promoter. These elements are many and varied and a small number are discussed below to illustrate important principles.

The CCAAT box

A common motif found in a number of promoters is termed the CCAAT box, an element which is found at variable distances and orientations with respect to the CAP site. A promoter may contain one or several of these elements e.g. the protein disulphide isomerase gene contains six CCAAT boxes located between -108 and -378 (Tasanen *et al.*, 1992).

In common with many transcriptional control elements the CCAAT box is capable of binding a range of different sequence-specific transcription factors. The first two CCAAT binding proteins to be identified were the CCAAT/enhancer binding protein C/EBP (Johnson *et al.*, 1987) and CCAAT transcription factor CTF/NF1 (Jones *et al.*, 1985). Both of these transcription factors are capable of interacting with the CCAAT box of the of the HSV tk promoter, however they have distinct properties and are members of unrelated transcription factor families. The C/EBP factor is heat stable and can also bind to the related sequence GCAAT with high affinity, in contrast CTF/NF1 is heat labile and fails to bind to this sequence (McKnight and Tjian 1986). There is a large family of C/EBP factors, each member can bind to this sequence, and a number of other factors can bind to this and related sequences (Dorn *et al.*, 1987).

The octamer element

Another common element is the octamer sequence which in common with the CCAAT box, can bind a range of different transcription factors. This element was originally identified in the histone H2B promoters and the immunoglobulin IgH

and Igκ promoters. These sequences are necessary for the lymphoid-specific expression of these genes (Wirth *et al.*, 1987; Falkner and Zachua 1984). Although these elements are important in the tissue-specific activity of a number of promoters and enhancers, this sequence also confers cell-cycle regulated expression on the histone H4 and H2B promoters (La Bella *et al.*, 1988; Fletcher *et al.*, 1987).

The GC box

The GC rich 'TATA less' promoters of a number of growth related genes often contain a number of 'GC boxes' within the first few hundred base pairs from the site of transcriptional initiation e.g. H-*ras* (Ishii *et al.*, 1986) and c-*myb* (Dvorak *et al.*, 1989), as well as a number of housekeeping genes such as hypoxanthine phosphoribosyl transferase (Kim *et al.*, 1986) and dihydrofolate reductase (Swick *et al.*, 1989). Several genes contain a single GC box upstream of a TATA box such as the adenovirus E1B (Schmidt *et al.*, 1989) and the NF1 (Ammendola *et al.*, 1990) promoters.

Although many of these elements function by binding the ubiquitous transcription factor Sp1, some sites may be targets for other transcription factors. For example, studies of the P450I A1 gene revealed the presence of a single GC box in the promoter termed basic transcription element. This element was capable of binding both Sp1 and another transcription factor termed basic transcription element binding protein (BTEB). This transcription factor repressed this promoter but was capable of transactivating promoters with multiple GC boxes, whereas Sp1 can activate promoters with a single GC box. (Imataka *et al.*, 1992). These transcription factors were found to be present in all cells tested but varied independently in level, suggesting that the activation of promoters containing GC boxes will be dependent on the relative affinities of individual GC boxes for different factors, the relative levels of these factors and the regulatory context of the element in a promoter.

2.2 (iii) Modularity of promoters

In the preceding discussion the elements are described in isolation. However, promoters consist of multiple elements (or modules), each of which has intrinsic activity but all are necessary for the wild type activity of the promoter. For example HSV tk promoter contains two Sp1 sites which surround a CCAAT box. All of these three elements are required for maximal activity, however each has intrinsic activity (Jones *et al.*, 1985).

The existence of multiple elements has important functional consequences. Firstly, with multiple elements a promoter can be controlled differently in a number of situations e.g. basal and induced activity of cytokine genes in macrophages (Grove and Plumb 1993). Another important consequence of multiple elements is that of synergy. This is defined as when a promoters activity resulting from a number of control elements is greater than the sum of the individual activities. This is an important mechanism of control as this allows a gene to be finely tuned, needing a whole range of transcription factors present and active to allow maximum transcription.

The most straight forward mechanism of synergy is that of co-operative binding. This has been demonstrated to occur in a number of cases. For example, two tandemly linked progesterone / glucocorticoid response elements (PRE/GRE) were placed upstream of a heterologous promoter. These elements conferred a synergistic response in the presence of the activated receptors. DNA binding studies *in vitro* demonstrated that the presence of one progesterone receptor increased the affinity of binding at the second site one-hundred fold (Tsai *et al.*, 1989). Although several cases of co-operative binding have been documented to-date, it appears that co-operative binding is the exception rather than the rule and that other mechanisms are necessary to explain transcriptional synergy. For example, a similar study of two oestrogen response elements could find no evidence of co-operative binding despite their synergistic activation of a minimal promoter (Mathurose *et al.*, 1990).

Other mechanisms are likely to involve the interaction of transcription factors with components of the preinitiation complex with multiple interactions stabilising the complex.

The third important consequence of the modularity of promoters is that the activity of an element is dependent on its context within the promoter. An interesting example of this is provided by the oesteocalcin gene. The promoter of this gene contains a vitamin D response element which overlaps with a consensus AP1 site. Activation of this gene can be achieved by treatment with vitamin D, however transfection of the cell lines with expression vectors containing c-*fos* and c-*jun* reduced basal and abolished induced transcription (Schule *et al.*, 1990a and b). This is in contrast to the effect of an AP1 site in most promoters which is to induce transcription under these conditions, illustrating the importance of the context of a sequence in determining its activity (Angel *et al.*, 1987). Similarly the rat α1 glycoprotein gene contains two C/EBP binding sites in its promoter. The distal element overlaps that of a glucocorticoid response element, and acts in an antagonistic manner in the presence of glucocorticoids. This same element contributes positively to the promoters activity in the absence of glucocorticoids. This example illustrates that the same element can mediate different activities under different conditions (Nishio *et al.*, 1993).

Clearly the activity of a promoter is determined by a number of elements, both positive and negative which act together to confer a specific spatial and temporal gene expression pattern on a gene. However, promoters are not the sole determinants of transcription control. Sequences removed from the immediate promoter region can have profound effects on transcription: these are termed enhancers.

2.2 (iv) Enhancers

Sequences further from the site of transcriptional initiation than the promoter

can exert effects on the initiation of transcription. These sequences are termed the enhancers, and are usually larger in size than the promoter elements usually ranging from 40-700 bp in length. These enhancers may be positive or negative (termed silencers) in action, may be tissue-specific (for example the B-cell specific immunoglobulin H chain enhancer, Mercola *et al.*, 1983), inducible (e.g. rat aminotransferase Reik *et al.*, 1991) or constitutive in nature (e.g. SV40 enhancer, Ondek *et al.*, 1988). Enhancers are characterised by their distance and orientation independent nature and have been found to activate transcription despite being located many kilobases upstream from the CAP site (Pinket *et al.*, 1987), downstream from the polyadenylation site (Campo *et al.*, 1983), within introns (e.g. Huang *et al.*, 1993), and even within the protein coding regions of genes (e.g. the Hepatitis B enhancer, Tognoni *et al.*, 1985).

The mechanism of enhancer activity is not fully understood, but is thought in many cases to involve the looping out of the intervening DNA between the promoter and the enhancer, allowing interaction of the proteins binding these *cis*-acting sequences and components of the preinitiation complex (Ptashne *et al.*, 1986). The transcription factor Sp1, which binds both promoter and enhancer elements, has been demonstrated to form higher order structures, looping out DNA between GC boxes via direct protein-protein interactions (Su *et al.*, 1991), illustrating that DNA looping can be mediated by sequence-specific transcription factors.

The distinction between promoter and enhancer elements is not clear, as many elements are found in both enhancers and promoters. The octamer motif originally defined in promoters (Harvey *et al.*, 1982) is also an important component in many enhancers e.g. the SV40 enhancer (Nomiyama *et al.*, 1987). Multimers of promoter sequences can have enhancer activity when placed upstream of a heterologous promoter (Ondek *et al.*, 1987), illustrating the functional similarity of these sequences. Both promoters and enhancers exert their effect via the binding of *trans*-acting sequence-specific DNA-binding proteins. These proteins

are discussed in the section 2.3.

2.2 (v) Position effects and higher order chromatin structure

If enhancers are position and orientation independent (section 2.2 (iv) why don't they promisciously activate many surrounding promoters *in vivo*? In contrast to yeast where the UAS are usually relatively close to their promoter, higher eukaryotes often have enhancers which can act at long distances from the promoter. For example *Drosophila Ubx* gene can be activated by an enhancer 35kb away (Qian *et al.*, 1991). In addition enhancers have been documented to activate more than one promoter (eg.the *Drosophila* yolk protein genes YP1 and YP2 are activated by the same enhancer in fat bodies and a distinct enhancer directs expression in the ovary; Logan 1989). That enhancers have the ability to act promiscuously has been clearly demonstrated by their ability to activate heterologous enhancers in artificial constructs (e.g. Huang *et al.*, 1993; Ondek *et al.*, 1987) and the success of 'enhancer trap' experiments in *Drosophila* (Bellen *et al.*, 1989).

One mechanism which may limit the activity of enhancers may involve higher order chromatin structure. The DNA of higher eukaryotes is organised into a number of discrete domains. The physical compaction may also serve to organise DNA into functional units of transcription, outside which the enhancers can have no activity. This is supported by nuclease studies which reveal that active genes are within a sensitive region usually 10-100 kb in length. The organisation of these domains is unclear but it has been suggested that they form anchored loops attached to the nuclear scaffold (reviewed Gasser and Laemmli 1987).

Presumably these domains must have boundaries and might have a specialised structure. One candidate for these boundary sequences are the matrix attachment or scaffold attachment regions (MAR/SAR). These sequences are defined biochemically as regions of DNA which are bound to the nuclear matrix after extraction of bulk chromosomal protein (Mirkovitch *et al.*, 1984). These sites

have been mapped to regions surrounding a number of genes (Gasser and Lammli 1987; Garrard 1990). These elements and a number of other interesting potential boundary elements have been tested in functional assays.

Stable transfections and transgenic mice have been used as a functional assay to test sequences for the ability to determine position independent expression (PIE). Clearly elements other than those which act only as a boundary are necessary to determine PIE, however these elements should insulate a gene from the repressive effects of the surrounding sequences.

The chicken lysozyme gene is flanked at the 5' and 3' boundaries of DNAse1 sensitivity by regions previously designated SARs. These elements ('A' elements) when flanking a reporter construct had no effect in transient transfections, but increased transcription 10-fold in stable transfectants (Stief *et al.*, 1989). Similarly, transgenic mice containing the lysozyyme promoter and enhancer elements flanked by these A elements showed that the expression level was correlated with the copy number of transgenes (Bonifer *et al.*, 1990).

Studies of the *Drosophila hsp70* genes have defined elements termed specialised chromatin structures (scs) which flank one of these genes and appear to demarcate boundaries of chromatin perturbation within this heatshock puff in salivary gland polytene chromosomes (Udvardy *et al.*, 1985). Both of these elements are characterised by a set of nuclease hypersensitive regions surrounding a protected region of 200-350 bp. These elements can insulate the *Drosophila* reporter gene *white* (whose expression results in the development of white eyes) from positive and negative euchromatic effects when they flank this gene, irrespective of the site of insertion of these constructs (Kellum and Schedl 1991). This element could also block the activity of an enhancer when placed between it and the promoter, however an MAR could not block transcription in this assay, although the lysozyme A elements could block enhancer activity suggesting that these boundary elements may have separable functions (Kellum and Schedl 1992).

2.3 Transcriptional regulatory proteins

In addition to the general transcription factors and RNA polymerase II, transcription is controlled by sequence-specific transcription factors. These factors are often composed of modules or domains with discrete functions. Many transcription factors have separable DNA binding and transcriptional activating domains; for example the yeast transcription factor GAL4 can be experimentally split into functional DNA binding and transcriptional activation domains (Brent 1985; Keegan *et al.*, 1986). A large number of transcription factors have been identified, and many belong to families which share sequence similarity over a domain which mediates DNA binding or dimerisation. A number of these motifs are discussed in section 2.3 (i).

2.3 (i) DNA binding and dimerisation domains

Helix-turn-helix

This motif was first identified in prokaryotic transcription factors (CI, CRO, CAP). These proteins bind DNA as a dimer and consequently the binding sites show dyad symmetry. Their structures were determined and these proteins recognised DNA sequences via an α-helix inserted into the major groove. Binding of the factor as a dimer places the recognition helices in an anti-parallel organisation such that each is aligned to half of the dyad symmetric site.

A specific conserved constellation of amino acids allowed identification of eukaryotic helix-turn-helix (HTH) like proteins, initially the yeast MATα1 and MATα2, and subsequently extended to the *Drosophila* homeotic genes *antennapedia, fushi tarzu* and *ultrabithorax* (Matthews *et al.*, 1982; Scott and Weiner 1984; Sheperd *et al.*, 1984). These genes which are involved in segmentation in *Drosophila* share extreme sequence relatedness across a 60 amino-acid domain - the homeobox. These transcription factors are also found in

mammalian cells, and bind to a sequence containing an AT-rich core. These proteins play a key role in embryogenesis and development.

Zinc fingers

This common motif was first discovered in transcription factor IIIA (TFIIIA) which binds to the internal promoter of the 5S rRNA genes. Cloning and sequencing revealed the presence of a repetitive motif cys-N2-cys-N12-his-N2-his. This motif was repeated nine times (Shastry *et al.*, 1982). Each of these repeats binds a zinc ion via a tetrahedral coordination of the cysteine and histidine residues. The intervening amino-acids loop out to form a "finger". This type of zinc finger motif is termed the C_2H_2 motif and this sub-family includes the Sp1 and TFIIIA transcription factors (Kadonaga *et al.*, 1987).

A related motif is termed the Cx zinc finger. This is typified by clusters of 4-6 cysteines often separated by 2 or 4 amino acids. These motifs occur in 25-30 amino-acid clusters that tend to be repeated. Members of this group include the hormone receptors, GAL4 and EF-1 (Ham and Parker 1989; Johnston 1987; Sargant *et al.*, 1990). As for the HTH proteins, the zinc fingers serve to present an α-helix which provides the binding specificity of the finger. This is a common feature of sequence-specific DNA-binding proteins.

Leucine zippers

The leucine zipper is a motif found in a number of transcription factors. The motif was first discovered in the CCAAT/enhancer binding protein or C/EBP (Landschulz *et al.*, 1988). This motif has now been found in a number of other proteins e.g. Myc, GCN4, ATF, Fos and Jun. The family is separated into two groups: the basic-leucine zipper (bZIP) which includes C/EBP and Fos, and other transcription factors which contain another dimerisation/DNA binding motif; these are the helix-loop-helix leucine zipper proteins which include Myc and Max (Blackwood and Eisenman 1991). The leucine zipper mediates dimerisation via a

coiled coil arrangement of parallel α-helices, in which a leucine occurs at every seventh residue. This dimerisation brings together the preceding basic regions necessary for sequence-specific binding (O'Shea *et al.*, 1989). The dimerisation is specific and the leucine zipper mediates this control (Kouzarides and Ziff 1989).

Helix-loop-helix

This sequence was first identified as a region of homology between the products of the *c-myc*, *myoD* and the *Drosophila achaete-scute* complex genes (Lassar *et al.*, 1989; Villares *et al.*, 1987). This motif has also been identified in the products of a number of *Drosophila* cell type determination genes e.g. *daughterless*, *hairy* as well as in the immunoglobulin enhancer binding proteins E12 and E47 (Murre *et al.*, 1989) .

Although there is no structural data the protein has been divided into two domains, the helix-loop-helix (HLH) and an adjacent basic region (Tapscott *et al.*, 1988). The HLH domain contains two stretches of amino acids capable of forming amphipathic α-helices (Murre *et al.*, 1989). The HLH domain has been demonstrated to be capable of mediating protein dimerisation (Murre *et al.*, 1989; Murre *et al.*, 1989b). The basic HLH structure is critical for the myogenic activity of MyoD and the transforming activity of Myc (Tapscott *et al.*, 1988; Weintraub 1991; Stone *et al.*, 1987). The activity of these proteins is critically controlled by the choice of dimerisation partner. This is discussed in more detail in section 2.4

Other classes of DNA-binding domains

A variety of other domains exist (eg Ets and POU (Le Prince *et al.*, 1983; Herr *et al.*, 1988) and new domains are being defined in the light of newly cloned transcription factors. A large number of the known factors fall into one of the above families, suggesting that a limited number of domains have been utilised by evolution to solve the problem of sequence-specific DNA-binding.

2.3 (ii) Transcriptional activating domains

The ability to experimently swap domains of transcription factors to generate chimeric proteins has suggested that in many cases the binding to DNA many serve a neutral function in terms of transcriptional activity (Brent 1985; Keegan *et al.*, 1986). In other words, the DNA-binding domain serves only to bring the activating domain into the region involved in the initiation of transcription.

In contrast to the conserved nature of DNA binding motifs, transcriptional activation domains appear to have little underlying structure in common. Three types of activating domain have been classified:

(i) Acidic activating domains - composed of stretches of negatively charged amino acids which may be able to form amphipathic α-helices , with negatively charged groups on one surface of the helix and hydrophobic groups on the other (Hope and Struhl 1986; Hope *et al.*, 1988).

(ii) Glutamine-rich domains are found in a number of transcription factors such as Sp1, OTF-2 , AP2 and SRF (Clerc *et al.*, 1988; Williams *et al.*, 1988; Norman *et al.*, 1988).

(iii) Proline domains are found in CTF and NF-1 transcription factors (Mermod *et al.*, 1989).

A protein may have more than one activating domain e.g. Sp1 contains four activating functions (Pascal and Tjian 1991). Some activating regions may require to be induced; e.g., the human oestrogen receptor has two transcriptional activating functions, one is constitutive and the other is induced by ligand binding (Tasset *et al.*, 1990).

Experimental evidence is available implicating a number of targets of transactivation domains. A number of mechanisms can be envisioned to explain how transcription factors mediate transactivation. Most transcription factors function via stabilising/facilitating preinitiation complex formation. Transcription factors have been demonstrated to interact with the basal transcription machinery

including TATA-binding protein (TBP), TAFs (TBP-associated factors) and TFIIB (Lin *et al.*, 1991; Ing *et al.*, 1992; Zhou *et al.*, 1992). The existence of a large number of TAFs or coactivators suggests that there may be a number of possible sites of interaction with transactivation domains of transcription factors. As mentioned in section 2.1, transcriptional activation can also be mediated by destabilisation of chromatin structure; other mechanisms may include protein-protein interactions which may allow enhancer binding proteins to be brought into close proximity. Evidence for the latter mechanism is that one of the four Sp1 transactivation domain can mediate DNA looping *in vitro* (Su *et al.*, 1991).

2.3 (iii) Dimerisation and the control of transcription factor activity

The majority of known transcription factors bind DNA as multi-protein complexes that frequently take the form of dimers. Many transcription factors are members of a family of related proteins which may have the ability to homodimerise and/or heterodimerise. This ability to dimerise is often an absolute requirement for transcription factor activity. This dimerisation is not promiscuous as even proteins which dimerise through the same class of domain cannot always form heterodimers. For example, although the C/EBP transcription factors and Fos and Jun are members of the bZIP super family, the C/EBP factors are unable to dimerise with either Fos or Jun.

Why is dimerisation so common within transcription factor families? A number of important regulatory consequences can be envisioned as a result of dimerisation and these are discussed.

DNA binding

The most obvious functional consequence of dimerisation is the alteration of DNA binding specificity. The widespread use of dimerisation provides an explanation as to why so many recognition sequences show dyad symmetry.

Homodimers would be expected to bind to sites showing dyad symmetry, whereas the recognition sites of heterodimers may be composite elements. An example of altered binding specificity depending on the choice of dimerisation partner is provided by c-Jun. This protein binds preferentially to an AP1 site when dimerised with itself or another member of the Fos/Jun family. Jun is also capable of dimerising with an ATF family member, and this heterodimer recognises the related cAMP element in preference to the AP1 site (Ivashkiv *et al.*, 1990; Benbrook and Jones 1990). In addition to altering the sequence specificity of transcription factors, dimerisation can also alter the affinity of a factor for its binding site. The AP1 family provides an example of this type of regulation: Jun-Jun homodimers bind the AP1 site with an affinity 500-fold less than Fos-Jun heterodimers, although this is a consequence of the greater stability of the Fos-Jun heterodimers (Cohen and Curran 1990). Similarly, heterodimers of MyoD and the E12/E47 proteins bind the κE2 site of the immunoglobulin κ gene with a higher affinity than homodimers (Murre *et al.*, 1989).

Negative regulation of transcription factor activity

A number of dominant negative regulators of transcription factor activity have been characterised. These factors seem to play a central role in transcriptional control particularly during differentiation.

The basic helix-loop-helix gene *myoD* can confer a muscle-specific phenotype on heterologous cells when its expression is driven by a viral LTR (see section 2.5). The levels of this factor do not however change during myogenesis and the regulation seems to be exerted by a protein termed Id. This protein lacks a basic domain and forms inactive heterodimers with E12/E47 and to a lesser extent MyoD (Benezera *et al.*, 1990). During myogenic differentiation the levels of Id fall, allowing the formation of functional MyoD-E12/E47 dimers which then activate a battery of muscle-specific genes. Id is not limited to muscle cells with Id or Id-like genes expressed in a range of tissues, including oesteoblasts, haemopoietic cells

and neuronal precursors, as well as displaying a broad pattern of expression during embryogenesis (Kawaguchi *et al.*, 1992; Duncan *et al.*, 1992; Wang *et al.*, 1992). Evidence that it plays a similar role in other systems is that over-expression in myeloid cells blocks differentiation (Kreider *et al.*, 1992).

These negative regulators are not restricted to the bHLH family of transcription factors, for example the C/EBP family contains a member CHOP-10 which does not contain a DNA-binding domain and can inhibit C/EBP activity on co-transfection (Ron and Habener 1992).

Regulation of transactivation capacity

An interesting example of the control of transcriptional activation properties via the choice of dimerisation partner is provided by the Myc/Max proteins. Max is present constitutively and readily forms homodimers which bind to E-box sequences (Blackwood and Eisenman 1991). These homodimers however fail to transactivate gene expression. As a result of a proliferation signal Myc is induced, this protein is unable to form homodimers but forms heterodimers with Max which transactivate gene expression via binding to E-box sequences (Amati *et al.*, 1992).

Other regulatory possibilities resulting from dimerisation include the ability to respond to different signal transduction pathways. For example C/EBPβ is IL-6 responsive, and heterodimer dimerisation with this factor can confer IL-6 induction on the heterodimer (Poli *et al.*, 1990).

2.3 (iv) The regulation of transcription factor activity by phosphorylation

There are four main levels at which phosphorylation can affect transcription factor activity.

1) Control of subcellular location.

2) Alteration of DNA binding activity.

3) Alteration of transactivation properties.

4) Choice of dimerisation partner.

The yeast transcription factor SW15 is required for cell-cycle regulated expression of the HO site-specific endonuclease. This transcription factor translocates to the nucleus during G1 of the cell cycle. The inactive cytoplasmic form is phosphorylated at three cdc2 consensus sites near or within the basic nuclear localisation signal (Moll *et al.*, 1991). Mutation of these three serine residues to alanine results in a constitutively active nuclear SW15 protein. The regulation is presumably mediated by cdc2 kinase or a similar cell cycle regulated kinase *in vivo*.

Other examples include the Rel family of transcription factors. NFκB when inactive is held in the cytoplasm by interaction as a result of dimerisation with IκB. *In vitro* phosphorylation of IkB by protein kinase A and C (PKA and PKC) results in activation of NFkB (Shirakawa and Mizel 1989; Ghosh and Baltimore 1990).

Phosphorylation of c-Myb by casein kinase II (CKII) *in vitro* results in a decrease in DNA binding, which is reversed by phosphatase treatment (Luscher *et al.*, 1990). Since these CKII sites are phosphorylated *in vivo* they may regulate DNA binding activity *in vivo*. This postulate is supported by the inability of CKII to inhibit v-Myb activity as a result of loss of these sites which is generated by a truncation necessary for the oncogenic activity of this protein (Luscher *et al.*, 1990). Another example is provided by the serum response factor (SRF), which binds to the serum response element in the c-*fos* promoter. In A431 cells this activity is partly inducible by epidermal growth factor (Prywes and Roeder 1986). Phosphorylation of recombinant SRF by CKII at one or more of a number of serines close to the DNA binding domain can stimulate DNA binding (Janknecht *et al.*, 1992). Whether this has a role *in vivo* is unclear, but microinjection of CKII into the cytoplasm led to an increase in SRF phosphorylation and c-*fos* transcription (Gauthier-Roviere *et al.*, 1991)

In most cases phosphorylation effects on transactivation are of positive control. The cAMP response element binding protein (CREB) mediates transcriptional activation of cAMP responsive genes upon phosphorylation by protein kinase A (PKA) (Gonzalez *et al.*, 1989). Most experiments rule out an effect of PKA on either DNA binding or dimerisation (Yamamoto *et al.*, 1989). The target of PKA phosphorylation of CREB is serine 133 and mutation of this residue abolishes transactivation activity (Gonzalez and Montmimy 1989).

The yeast transcription factor ADR1, which increases the expression of the ADH2 gene via an upstream activating sequence, provides an example of negative regulation of transactivation by phosphorylation. Catabolite repression of ADR1 activity requires PKA and appears to be due to direct phosphorylation of ser-230 (Cherry *et al.*, 1989). Equivalent ADR1 DNA binding activities are detected in repressed and non-repressed cells and a constitutively active mutant has an altered PKA consensus for phosphorylation suggesting the inhibition is at the level of transactivation (Taylor and Young 1990).

There is no direct evidence for the control of dimerisation by phosphorylation, however as the choice of dimerisation partner has profound functional consequences, this seems an attractive level to control transcription factor activity. Under certain conditions C/EBPβ is phosphorylated *in vivo* at a serine residue within the leucine zipper domain. This phosphorylation does not alter the DNA binding properties but increases the transactivation activity of this factor, suggesting that this phosphorylation may alter affinity for different heteromeric partners although other mechanisms could explain these results (Wegner *et al.*, 1992).

Phosphorylation can alter the activity of a single transcription factor both positively and negatively, the best example being provided by c-Jun. Phosphorylation at one set of sites negatively regulates DNA binding, whereas phosphorylation at another set of sites increases transactivation (reviewed in Hunter and Karim 1992). A single exogenous signal can regulate phosphorylation at a

number of sites coordinately, for example TPA can induce dephosphorylation of the inhibitory sites in c-Jun and induce phosphorylation of the stimulatory sites (Pulverer *et al.*, 1992).

The role of phosphatases is also likely to be of key importance in the regulation of transcription factor activity, e.g. it has been demonstrated that transcriptional attenuation following cAMP induction requires protein phosphatase 1 mediated dephosphorylation of CREB at serine 133 (Hagiwara *et al.*, 1992).

The importance of phosphorylation is underlined by the fact that almost every transcription factor studied is a phosphoprotein. However, in most cases the functional consequences of this phosphorylation is unknown.

2.4 Tissue-specific gene expression

A number of tissue-specific genes have been characterised in some detail for a range of different lineages. These studies have characterised both the *cis*-acting elements which confer this tissue specificity and the factors which bind them to mediate it. Liver-specific gene regulation has been studied in some detail and has provided a number of insights into the mechanisms controlling tissue-specific gene expression.

The serum albumin gene is expressed exclusively in the liver and its regulatory elements have been dissected in detail. The promoter of this gene contains five *cis*-acting motifs in addition to the TATA box. These functionally defined elements have been well conserved throughout mammalian evolution. Each of these elements binds transcription factors present in liver nuclei (HNF1, NFY, C/EBP, DBP and an NF1-related protein). With the exception of the CCAAT motif, each of these sequences contributes to the hepatocyte specificity of the promoter (Maire *et al.*, 1989; Cereghini *et al.*, 1987; Gorski *et al.*, 1986). This modular organisation is typical of promoters of other liver-specific genes, with a number of elements having intrinsic activity. In addition, many of the genes also

contain elements which bind ubiquitous factors. These elements do not contribute to tissue specificity, but can increase the level of transcription.

Enhancers as well as promoters are important in the control of liver-specific gene expression. The serum albumin gene in addition to its tissue-specific promoter also has a liver-specific enhancer. This enhancer is located 10kb upstream and transgenic mice revealed that this element mediates expression exclusively in the liver (Pinkert *et al.*, 1987) and contains three functionally defined elements, binding NF1, C/EBP and HNF3 family members (Herbst *et al.*, 1989; Jackson *et al.*, 1993). Similarly the liver-specific α-1 microglobulin/bikunin gene displays a modular structure. This enhancer contains six functional elements which bind members of the HNF1, HNF3 and HNF4 families of transcription factors. In contrast to the serum albumin gene the tissue-specificity seems to be conferred by the enhancer alone (Rouet *et al.*, 1992).

The simplest explanation of how transcription factors control tissue-specificity of gene expression would be that specific regulatory elements are bound by transcription factors which are restricted to a particular cell lineage. However, to date no transcription factor has been discovered whose expression is restricted solely to the liver. It seems that liver-specific promoters and enhancers are bound by a range of factors which are enriched in liver cells compared to many other tissues. A number of transcription factor families have been identified which are important in transcriptional regulation in liver cells. These include C/EBP, hepatocyte nuclear factors 1, 3 and 4 (reviewed in Lai and Darnell 1991). These factors are expressed at high levels in the liver, but not exclusively. e.g. HNF1α is expressed in the liver and has been demonstrated to bind to the 5' region of a number of liver-specific genes and activate their transcription in co-transfection assays e.g. serum albumin (Schorpp *et al.*, 1988; Cereghini *et al.*, 1988), aldolase B (Tsutsumi *et al.*, 1989). However, HNF1α is also expressed in kidney and lung, although kidney and liver express a number of genes in common e.g. α1 antitrypsin which contains an HNF1 site within its promoter (Baumhueter *et al.*, 1988). Clearly single

elements are not tissue-specific, rather the combination of a number of *cis*-acting sequences within a promoter or an enhancer together confer tissue specificity on a gene.

Further complexity is emerging in the regulation of tissue-specific gene expression, with the discovery of tissue-specific co-activators. The HNF1α homeodomain protein can dimerise with itself or HNF1β, but maximal transactivation requires a novel cofactor termed dimerisation cofactor of HNF1 (DcoH) which does not bind DNA. This factor shows a tissue-specific distribution with levels high in the liver, and can increase transcription up to 200-fold. The mechanism of action is unclear but this factor cannot bind DNA, rather it acts via binding HNF1 and stabilising dimers (Mendel *et al.*, 1991).

A similar example of a co-activator playing a role in tissue-specificity is provided by B-cell specific activation via octamer elements. These motifs have been demonstrated to be important in mediating the B-cell specific expression of the immunoglobulin genes and paradoxically the cell cycle regulated expression of the histone H2B genes. Until recently it was presumed that these two effects were mediated by separate octamer binding proteins, i.e. the tissue-restricted Oct-2 was responsible for the immunoglobulin gene expression and the ubiquitous Oct-1 responsible for H2B gene expression. Recent work has implicated a novel B-cell specific co-factor OCA-B in the tissue-specific activity of octamer elements. This factor was isolated by testing fractions from B-cells for the ability to restore IgH chain transcription *in vitro* upon a HeLa extract. This factor can activate the IgH promoter in conjunction with either Oct-1 or Oct-2 (Luo *et al.*, 1992).

Interestingly, this co-activator had no effect on the octamer/Oct-1 dependent transcription of the histone H2B promoter. The reason for the promoter-specific effect of this factor is unclear, but may be a result of the short and highly conserved spacing between the octamer and TATA elements in H2B promoters (Harvey *et al.*, 1982). This shorter spacing may obviate the need for a co-activator, further illustrating the importance of the context of an element in determining activity.

2.5 Transcription factors and the control of differentiation

The *myoD* gene provides a paradigm for the role of transcription factors in development and differentiation. This gene is capable of activating previously silent muscle-specific genes when introduced into a large variety of cell types under the control of a viral LTR. *MyoD* is a member of a family of muscle-specific HLH proteins (*myogenin, myf5, mrf-4-herculin*) which all seem to play a similar role in activating muscle-specific genes (and each other). A positive feedback mechanism is generated by the ability of MyoD to activate both its own expression and the other members of the *myoD* gene family which leads to muscle differentiation.

MyoD dimerises with the ubiquitous HLH proteins E12 and E47 to form active heterodimers. The levels of these three HLH proteins remain steady during differentiation. The control seems to be exerted by the negative regulator Id (see section 2.4 (ii). This protein readily forms dimers with E12 and E47 which are unable to bind DNA (Id lacks a basic domain). The level of this repressor falls during myogenic differentiation (Benezera *et al.*, 1990), allowing the formation of MyoD-E12/47 heterodimers which mediate the muscle-specific differentiation program.

Clearly those cells which contain MyoD are committed to muscle differentiation. This begs the question: how is the decision to continue to proliferate or to differentiate controlled? Id is expressed at high levels in proliferating cells and is down regulated on withdrawal of serum. Hence in conditions favouring proliferation the levels of Id are high and this prevents the E12/47 proteins from dimerising with MyoD. This is supported by the fact that the majority of the E12 and E47 proteins are complexed in inactive heterodimers with Id in proliferating myoblasts (Jen *et al.*, 1992). However proliferation signals regulating Id levels do not alone control the differentiation program. In contrast to serum, which inhibits the DNA-binding activities of MyoD and Myogenin and induces Id expession, TGFβ inhibits the myogenic activity of these factors without affecting their ability to

bind to DNA, hence excluding a role for Id in TGFβ mediated repression of differentiation (Brennan *et al.*, 1991).

Recent work has provided strong evidence for the role of the product of the retinoblastoma susceptibility gene (pRb) in muscle cell commitment and differentiation (Gu *et al.*, 1993a). This work demonstrated that pRb was required for the growth arrest and differentiation often observed when the *myoD* gene is introduced into a number of cell types. Two oesteosarcomas cell lines Saos-2 and USOS were used as experimental system. These cell lines are phenotypically similar but Saos-2 lacks a functional pRb gene. Expression vectors containing *myoD* in the sense or antisense orientation were introduced into these cell lines. The inclusion of the *myoD* in the expressing vector reduced the number of colonies produced in the USOS (pRb+) by 50% in comparison to the numbers generated using the antisense construct. Introduction of these constructs into Saos-2 (pRb- cell line) showed no reduction in colony formation. Stably transfected Saos-2 cell lines expressing *myoD* were retransfected with Rb; this resulted in the cessation of proliferation and the expression of the muscle-specific myosin heavy chain. Hence Rb is required for both growth arrest and muscle-specific differentiation.

MyoD and pRB interact directly both *in vitro* and *in vivo*; however, at least *in vitro*, MyoD could only bind to the underphosphorylated form of pRb. As pRb remains in this underphosphorylated form in differentiating cells (even in response to growth factors) suggesting that MyoD may lock pRb in this growth factor unresponsive state. The correlation of differentiation with the inability to rephosphorylate pRb is seen in other terminally differentiating systems including HL60 cells (Whyte and Eisenman 1992; Mihara *et al.*, 1989). The key role of pRb in inhibiting proliferation was demonstrated by the ability of differentiated muscle cells containing a *ts* SV40 large T antigen to re-enter the cell cycle when large T was activated (and thus inactivating the pRb protein). The pRb protein also seems to stabilise the MyoD-E protein heterodimers, as when pRb is removed from muscle cell extracts E-box activity is diminished or abolished. This suggests that pRb aids

in myogenesis by increasing the number of functional myogenic heterodimers.

The question remains, why does the 'free' MyoD in myoblasts not interact with pRb in G1 (when pRb is underphosphorylated) and commit cells to differentiation? An intriguing possibility is suggested by the demonstration that c-Jun can bind MyoD (Bengal *et al.*, 1992). This results in mutual inactivation of AP1 and MyoD activity. A further link demonstrating the antagonistic action of MyoD on AP1 is that MyoD has been demonstrated to down regulate the c-*fos* promoter (Trouche *et al.*, 1993). Serum and TGFβ (which does not increase Id expression) increase the levels of Fos and Jun; these oncogenes in either viral or cellular form can block differentiation when over-expressed in myoblasts (Brennan *et al.*, 1991; Lassar *et al.*, 1989). This along with the evidence that the level of MyoD (or other myogenic transcription factors) is important in determining the proliferation/differentiation choice suggests that the relative levels of these factors will determine the choice of proliferation or differentiation. This dual role of pRb in cell-cycle control and muscle-specific differentiation provides a molecular mechanism for the incompatibility of proliferation and terminal differentiation. As differentiation in this system requires pRb in an underphosphorylated form to interact with MyoD, whereas hyperphosphorylated pRb is necessary for cell cycle progression. It is interesting to note that induction of differentiation of HL60 cells results in a rapid induction of the product of the *waf*-1 gene (Hongping *et al.*, 1994; Steinman *et al.*, 1994). This gene encodes a cyclin-dependent kinase inhibitor whose activation results in the prevention of pRb phosphorylation (Xiong *et al.*, 1993; Harper *et al.*, 1993; El-Deiry *et al.*, 1993), and may be an important mediator of differentiation in a number of systems.

The CCAAT / enhancer binding protein α is expressed in a number of tissues, including lung, liver, adipose, gut and placenta at varying levels. Several lines of evidence suggested C/EBPα may have an important role in terminally differentiated tissues. In adult liver C/EBPα is restricted to the nuclei of mature hepatocytes (Birkenmeier *et al.*, 1989), whereas proliferating hepatoma cells

contain very low levels (Friedman *et al.*, 1989). Similarly, cultured 3T3-L1 adipoblasts do not express C/EBPα when proliferating, but exhibit marked induction when stimulated to differentiate (Birkenmeier *et al.*, 1989; Christy *et al.*, 1989). This correlation suggested that C/EBPα may have a role in differentiation, by switching on a differentiation program and/or being involved in the maintenance of a terminally differentiated phenotype. Evidence for the latter is that C/EBPα is involved in the regulation of a number of genes expressed only in specific terminally differentiated tissues, as well as being implicated in many others. These include the hepatocyte-specific serum albumin gene (Friedman *et al.*, 1989) and the adipocyte-specific 422(aP2) and GLUT4 (Christy *et al.*, 1989; Kaestner *et al.*, 1990).

C/EBPα also appears to be a key mediator of terminal differentiation in adipocyte tissue and perhaps in a number of other tissues also. 3T3-L1 preadipocytes with appropriate hormonal stimulation can differentiate into cells with morphological and biochemical characteristics of normal adipocytes (Green and Kehinde 1974, 1975 and 1976). Using this as a model system a number of workers have demonstrated that C/EBPα antisense constructs inhibit the differentiation of these cells as measured by the lack of expression of a number of adipocyte-specific genes and the accumulation of cytoplasmic triglyceride (Lin and Lane 1992; Samuelson *et al.*, 1991). Conversely the activation a conditional fusion protein C/EBPα-oestrogen receptor with estradiol led to a direct cessation of growth which was dependent on the C/EBPα portion of the protein for activity. When abetted by the effects of three adipogenic hormones this chimeric protein could promote terminal differentiation (Umek *et al.*, 1991).

c-Myc is tightly correlated with proliferation and constitutive expression of the gene in a number of tissues can block differentiation. This includes adipogenesis in which constitutive expression of c-*myc* prevent 3T3-L1 adipoblasts from differentiating. This enforced expression of c-*myc* prevented the normal induction of C/EBPα in response to differentiation signals. Transfection of these cells with

C/EBPα-expressing constructs led to a reduction in both the number of neomycin resistant colonies formed (in comparison to the number generated with an antisense construct) and an increase in the number of cells containing lipid droplets in their cytoplasm (Freytag and Geddes 1991). This suggests that c-Myc negatively regulates the expression of the C/EBPα gene and as a result inhibits the differentiation of these cells.

As C/EBPα is present in a number of terminally differentiated tissues, most notably the liver, it seems likely that it may play a similar role in a number of tissues i.e. growth arrest and the promotion of a predetermined differentiation program. This is supported by the ability of the C/EBP-ER fusion protein to produce growth arrest in a number of different cell lines including the pluripotent 10 $t^{1/2}$ cells.

There are a number of similarities between C/EBPα and MyoD. Both produce growth arrest when expressed at high enough levels to overcome proliferative signals (Umek *et al.*, 1991; Sorrentino *et al.*, 1990) and both are involved in the expression of genes necessary for a specialised phenotype. However, unlike MyoD, C/EBPα cannot confer a specific differentiation program when over expressed in heterologous cell lines. This is not suprising as C/EBPα is expressed in a number of different tissues, but may act as a switch between proliferation and differentiation depending on the relative strengths of proliferation and differentiation signals, and as an auxiliary factor in a predetermined differentiation pathway.

Although the above provides an attractive model for the control of differentiation it is an over simplification. A range of different C/EBP family members exist, which are coexpressed at varying levels in different tissues. These family members can heterodimerise in all intra-familiar combinations tested (Poli *et al.*, 1990; William *et al.*, 1991; Cao *et al.*, 1991). Interestingly a C/EBP family member has been cloned (CHOP-10) which has two prolines in the highly conserved basic domain. This protein is capable of acting as a dominant negative

inhibitor of C/EBP activity as measured by transient transfection studies (Ron and Habneur 1992). This gene is induced in parallel with C/EBPα during adipogenesis however there is still a large increase in C/EBP activity in these terminally differentiated cells. Whether CHOP-10 is simply a negative regulator of C/EBP activity in terminal differentiation is not known. Similarity between the mouse CHOP-10 and the hamster GADD 153 gene suggests they may act in a functionally similar manner. The GADD 153 gene is induced on DNA damage and is associated with growth arrest of the cells, suggesting that perhaps CHOP-10 has a role in the C/EBPα mediated growth arrest (Bartlett *et al.*, 1992).

The pattern of C/EBPα expression, high in terminally differentiated tissues and low in proliferating cells is not absolute, the reciprocal pattern is true in the myeloid system with C/EBPα and C/EBPδ being highest in immature proliferating cells and C/EBPβ highest in the terminally differentiated cells (Scott *et al.*, 1992). It may be that the varying isoforms from tissue to tissue are involved in defining a specific pattern of gene control and perhaps a differentiation program unique to that tissue (the role of C/EBP in myeloid gene control is discussed in section 3.).

Chapter 3: The transcriptional control of myelopoiesis

Gene regulation during myeloid differentiation

During haemopoiesis, pluripotent stem cells differentiate to generate precursor cells committed to a particular lineage, which eventually mature into functional, morphologically distinct end cells. This process requires the co-ordinate regulation of a number of genes which can be into a number of groups: the ubiquitous 'housekeeping' genes, those necessary for proliferation, differentiation or survival and those encoding proteins specifically required for the function or maintenance of the fully differentiated cell.

The existence of a large number of myeloid cell lines (c.f. section 1.5), a number of which can be induced to differentiate *in vitro*, has provided a useful homogenous source for the cloning of myeloid-specific genes and systems in which to study their regulation. Studies of this kind have been undertaken to identify *cis*-acting sequences which confer both myeloid and differentiation-stage-specific expression on genes, and to characterise the *trans*-acting factors which bind at these sites. Although a large number of studies have identified genes which are expressed exclusively in myeloid cells or in a differentiation-stage specific-manner, detailed studies identifying the transcriptional control elements are available only for a small number of genes.

The chicken lysozyme gene is constitutively expressed in macrophages (Hauser *et al.*, 1981). The actively transcribed gene is located in a 20kb DNAseI sensitive region. This region is flanked by the so called 'A' elements (discussed in section 2.1 iii), which together with the -6Kb enhancer can confer high level position independent gene expression on reporter constructs. This enhancer is hypersensitive in cells actively transcribing the lysozyme gene (or have this potential). Deletion analysis has narrowed this enhancer down to 157bp, and this is sufficient to confer tissue-specific activity. Footprinting studies identified at least six

elements which bound transcription factors, however nuclear extracts from non-myeloid cells also contained factors which bound at a number of sites. Surprisingly overlapping oligonucleotides containing at least two of these sites all contributed to the specificity of the enhancer when cloned upstream of the HSV tk promoter. These elements show sequence similarity to AP1, NF1 and octamer binding sequences although no attempt to identify the interacting proteins was reported in the study (Grewal *et al.*, 1992).

In contrast, the mouse genome contains two lysozyme genes, the M and P genes expressed in myeloid and paneth cells respectively. The two genes are the result of a duplication event, and the downstream M gene contains only 1.7Kb of 5' flanking sequence (Cross and Renkawitz 1990). Analysis by nuclease sensitivity of the surounding chromatin revealed a complex pattern of hypersensitive sites occurring at different stages of differentiation in myeloid cell lines Whereas no nuclease sensitivity was found in non-myeloid cells. (Mollers *et al.*, 1992). The 5' flanking region of this gene was not sufficient to confer activity on a heterologous promoter. However, a 3' region (unique to the M gene) encompassing a cluster of hypersensitive sites could confer strong enhancer activity on this construct in all cell lines tested. Further investigation revealed that this region was under methylated in expressing cells. Methylation of a single CpG residue in the enhancer core was only observed in non-expressing cells, and this residue was demethylated when FDC-P A4 cells were induced to differentiate *in vitro*. Binding of a nuclear factor to this site was inhibited by methylation, as was enhancer function. Demethylation was found to parallel or just follow the establishment of a hypersensitive site at this sequence. Treatment of immature myeloid cells with 5-azacytidine resulted in demethylation at this site accompanied by the activation of this gene; however this treatment failed to elicit a response in fibroblasts indicating the importance of other sequences in contributing to the tissue specificity of this gene (Klages *et al.*, 1992).

The CD11b gene encodes a subunit of the complement receptor type 3. The expression of this gene is limited to myelomonocytic cells and the rate of

transcription of this gene increases markedly during differentiation of myeloid cell lines towards monocytes/granulocytes (Pahl *et al.*, 1992). Although sequences further upstream contribute to the activity of the promoter, the first 92bp are sufficient to direct tissue-specific expression of a reporter gene. The transcription factor PU.I which has a limited tissue distribution, binds to a sequence around -20 *in vitro*. Mutation of this site reduced activity in U937 cells 3 to 4-fold, but had no activity in the epithelial cell line HeLa (Pahl *et al.*, 1993). An Sp1 site was also present in this fragment and *in vivo* footprinting of this site established that it was bound only in expressing cells. Mutations at this site reduced activity 18-fold, suggesting that PU.I may facilitate the binding of Sp1 in myeloid cells *in vivo*. A similar case of PU.I acting as a recruiting factor is seen at the immunoglobulin κ 3' enhancer (Pongubala *et al.*, 1993). These studies implicated Sp1 and PU.I in the tissue-specific activity of the CD11b promoter; however, those sequences which confer elevated expression on this gene as a result of differentiation were not identified although Sp1 was eliminated by mutational analysis.

These examples are of genes expressed in both monocytic and granulocytic lineages. Only one gene which is expressed exclusively in granulocytes has been studied in detail; this is the chicken mim-1 gene. This gene was originally isolated by differential hybridisation of ts v-*myb* transformed cells at permissive and non-permissive temperatures (Ness *et al.*, 1989). This gene encodes a secretable protein stored in the granules of both normal and v-*myb*-transformed promyelocytes. The promoter contains three sites which can bind v-Myb *in vitro* and the endogenous gene can be transactivated in HD11 chicken macrophage by E26 v-Myb, but not in non-myeloid cell lines. Transactivation studies have implicated Ets-2 in activating this gene synergistically with c-Myb, although whether the endogenous gene would be activated is unclear. Interestingly, only the E26 v-Myb (which is the product of a *myb-ets* fusion gene) and not the AMV v-Myb (which is not) could activate the endogenous gene in these cells.

Two other studies have demonstrated that C/EBP transcription factors can

synergistically activate the *mim-1* gene in conjunction with c-Myb. Two potential sites have been identified which bind the chicken homologue of C/EBPβ *in vitro* (Ness *et al.*, 1993). Mutation of one of these sites prevents transactivation by c-Myb. More surprisingly, expression of C/EBP transcription factors in fibroblasts could confer c-Myb inducibility upon the endogenous *mim-1* gene. C/EBPα or C/EBPδ but not C/EBPβ could also confer c-Myb inducibility on the lysozyme gene in these cells, suggesting that the C/EBP transcription factors may be important mediators of myeloid-specific gene expression (Ness *et al.*, 1993; Burk *et al.*, 1993).

The chicken C/EBPβ (NF-M) is also involved in the regulation of the chicken myelomonocytic growth factor. However, Myb is not required for the activity of this gene, but the myeloid-specific promoter contains two binding sites for this factor which act synergistically with a kinase inducible element which binds an AP1-like transcription factor (Sterneck *et al.*, 1992).

3.2 Transcription factors involved in myelopoiesis

The previous section discussed the regulation of genes which are myeloid-specific and/or are regulated in a differentiation-specific manner. What are the transcription factors responsible for this expression ? To date no transcription factor has been identified which can confer a myeloid-specific phenotype when expressed in heterologous cell types, nor has any factor been isolated which is strictly limited to the myeloid compartment. Despite this lack of obvious candidates for mediating myeloid-specific expression a number of transcription factors which have limited distribution patterns have been implicated in determining specific phenotypes within the myeloid lineages. Three families of transcription factors which are of particular relevance to this work are discussed in more detail in sections 3.3, 3.4 and 3.5. A number of other transcription factors merit a brief mention.

A key role for the helix-loop-helix proteins in granulocytic differentiation

has been illustrated by the ability of Id to block differentiation of the mouse myeloid progenitor 32D cell line when treated with G-CSF (Kreider *et al.*, 1992). This result implies the existence of HLH proteins in these cells which must become active to promote differentiation. The identification of the HLH proteins present in these cells will be particularly instructive given the role of cell-specific HLH proteins in other differentiating systems. The importance of these HLH proteins in other haemopoietic compartments is illustrated by the involvement of a number of these genes in lymphoid leukaemias (reviewed in Green and Begley 1992). A role for the AP1 transcription factor in monocytic differentiation has been suggested by a number of studies, although exactly which members of the Fos/Jun family are active in this complex in myeloid cells is unknown. The introduction and expression of c-*fos* and c-*jun* into M1 myeloblasts results in increased spontaneous differentiation (Lord *et al.*, 1993). Similarly, Egr has been implicated in inducing differentiation along the monocytic pathway (Nguyen *et al.*, 1993).

3.3 The Myb family of transcription factors

The c-*myb* proto-oncogene is the cellular homologue of the transforming gene of the avian myeloblastosis and the E26 (*myb-ets* fusion) viruses (Klempnauer *et al.*, 1982). The involvement of these viruses in transforming haemopoietic cells, and the high levels of c-Myb present in immature progenitors suggests that this protein may play a key role in the control of haematopoiesis (Westin *et al.*, 1982). This has been conclusively demonstrated using knockout mice, nullizygous for c-*myb*. These mice develop normally for 14 days then display a severe impairment of both erythropoieisis and myelopoiesis. Interestingly embryonic haematopoiesis in the yolk sac is apparently normal suggesting that other members of the Myb family may carry out overlapping functions (Mucenski *et al.*, 1991).

As well as c-*myb*, there have been two other cellular *myb*-related genes identified to-date, A-*myb* and B-*myb* (Normura *et al.*, 1988). In addition several

Myb related proteins have been described in species as diverse as *Drosophila*, yeast and *Zea mays*, with the DNA binding domain displaying the most homology (Peters *et al.*, 1987; Tice-Baldwin *et al.*, 1989; Paz-Ares *et al.*, 1987). All of these Myb related proteins are sequence-specific DNA binding proteins. The A- and B-Myb proteins have a wider tissue distribution than c-Myb which is limited to the haematopoetic system. A-*myb* is highly expressed in the kidney, colon and lymphoid tissue, whereas B-*myb* was expressed in all cell lines tested (Normura *et al.*, 1988).

3.3 (i) Functional domains of c-Myb

The c-Myb protein can be divided into three functional domains: the DNA-binding domain, the transactivation domain and the negative regulatory domain. The DNA-binding domain is the most conserved region of the protein and consists of three imperfect repeats of 50-52 amino acids; however, only two of these repeats are necessary for DNA binding. Sequence comparison suggests that each repeat may consist of three α–helices with homology to the HTH motif (Frampton *et al.*, 1989). Both mutational analysis and Raman spectroscopic analysis support this model structure (Frampton *et al.*, 1991; Grabrielsen *et al.*, 1991; Saikumar *et al.*, 1990; Kanei-Ishii *et al.*, 1990).

The transactivation domains have been functionally mapped for both c-Myb and v-Myb. This has identified a 50-residue domain, located C-terminal to the DNA-binding domain (Klempauer *et al.*, 1989; Sakura *et al.*, 1989; Ibanez and Lipsick, 1990). This transactivation domain is hydrophilic and slightly acidic, although it is not clear whether this is necessary for its activity (Weston and Bishop, 1989).

The presence of a negative regulatory domain is suggested by mutational analysis which has demonstrated that deletion of the N-terminus of c-Myb increases its transactivation up to 10-fold. Studies mapping this negative regulatory domain

have produced conflicting results. Dubendorf and co-workers demonstrated that two separate negative domains need to be deleted before transactivation by c-Myb increases (Dubendorf *et al.*, 1992). One of these domains was capable of inhibiting transcriptional activation in *trans* by Myb, Myb-VP16 or LEXA-Myb proteins. This inhibition is presumably mediated by protein-protein interactions and does not require DNA binding. A similar study identified a leucine zipper sequence in one of the negative regulatory domains. Disruption of this leucine zipper increases both transactivation and transforming activities of c-*myb*. This result suggests a protein interacts with c-Myb to repress its activity, and this leucine zipper could mediate this interaction with other as yet unidentified protein(s) (Kanei-Ishii *et al.*, 1992). A study has obtained partial sequence of two proteins which interact with this leucine zipper, although the effect of these interactions are unknown (Favier and Gonda 1994).

3.3 (ii) Genes regulated by c-Myb

There have been several attempts to identify c-Myb and v-Myb regulated genes; however, there is compelling evidence only for one target. This is the chicken *mim-1* gene which is directly regulated by both v-Myb and c-Myb. The promoter of this gene contains three Myb binding sites (Ness *et al.*, 1989). Other potential targets include the c-*myb* and c-*myc* genes as their promoters contain potential Myb binding sites and synthetic reporter constructs can be transactivated by c-Myb (Evans *et al.*, 1990; Zobel *et al.*, 1991; Nicholaides *et al.*, 1991). In transfection assays, c-Myb can activate gene expression without binding to DNA, as is the case for the activation of the hsp70 gene (Klempnauer *et al.*, 1989; Kanei-Ishii *et al.*, 1994). Presumably this action is mediated by binding negatively acting transcription factors, or perhaps by a 'squelching' mechanism titrating out a subset of co-activators necessary for transactivation by a number of transcription factors (reviewed Gill *et al.*, 1992).

3.3 (iii) Regulation of c-Myb activity

Alternative splicing of c-*myb* has been documentented in both human and murine cells, with a larger message generated by the inclusion of an extra exon located between exons 9 and 10 (Ramsey *et al.*, 1989; Dudek and Reddy 1989). In addition another spliced variant has been identified in human cells which included a cryptic exon located between exons 10 and 11. The messages generated by the alternative splicing events are similar in both human and mouse cells, the proteins generated are very different. In the murine cells the spliced variant generates a larger form of c-Myb (Ramsey *et al.*, 1989), whereas the human variants are predicted to generate 3' truncated proteins as a result of the inclusion of a stop codon in these exons (Sheng-Ong *et al.*, 1990). The functional consequences of these variants are unknown but the human proteins have similar 3' truncations as the viral Mybs.

Phosphorylation may also be an important mediator of Myb activity as specific interaction with a Myb binding site *in vitro* is abolished when c-Myb is phosphorylated at an authentic *in vivo* phosphorylation site (Luscher *et al.*, 1990). This site is lost in most oncogenic variants suggesting that this may uncouple Myb from its normal regulation.

3.3 (iv) C-Myb : role in proliferation and differentiation

Antisense and knockout experiments have demonstrated the importance of c-Myb in normal haematopoiesis. The importance of c-Myb in proliferation can be demonstrated with antisense oligonucleotides which inhibit growth of cell lines (Calabretta *et al.*, 1991; Venturelli *et al.*, 1990). Evidence suggesting that c-Myb is involved in differentiation includes the of ability c-Myb to block differentiation when over-expressed in MEL cells (Clarke *et al.*, 1988), whereas an alternatively

spliced variant enhances differentiation (Weber *et al.*, 1990). These experiments suggest that Myb may have a role in the regulation of differentiation, however it is difficult determine if these are a direct effect on differentiation rather than a consequence of stimulating or inhibiting proliferation. A number of lines of evidence suggests that Myb in addition to stimulating proliferation has a role in differentiation.

The most straightforward evidence that Myb is involved in determining a specific differentiation program is that it has been demonstrated to regulate the *mim-1* gene. It is extremely unlikely that this gene is involved in the control of proliferation, rather it seems to be a tissue-specific protein, necessary for the function of the neutrophil. Perhaps the most convincing evidence that this gene has a central role in haemopoietic differentiation is provided by studies with the viral forms of this protein. Avian macrophages can be transformed with either v-*myc* or v-*myb*, however only *v-myb* confers an immature phenotype on the transformed cells. Superinfection of v-myc transformed macrophage with a v-*myb*-eostrogen receptor construct confers an immature promyelocyte-like phenotype on the cells in the presence of estradiol, illustrating the dominance of Myb in this system in conferring a specific differentiation phenotype on these cells (Ness *et al.*, 1987; Burk *et al.*, 1991).

In normal myelopoiesis, c-Myb may maintain an early granulocyte gene expression program. Cells in which c-Myb is down regulated would enter a default pathway and become monocytic. Supporting evidence of this model is that c-*myb* and *mim-1* are co-expressed in normal promyelocytes and transformation of v-*myc* transformed macrophages with c-*myb* can transactivate *mim-1* (Nakano *et al.*, 1992). The E26-transformed cells resemble myeloblasts and express *mim-1*, whereas AMV-transformed cells resemble monoblasts and do not express *mim-1*. The AMV v-*myb* gene contains a number of point mutations relative to both c-*myb* and E26 v-*myb*. Back mutation of any one of three mutations in the DNA binding domain restores the ability to activate *mim-1* expression, and back mutation of two

of these mutations causes the transformed cells to resemble promyelocytes and cause promyelocytic leukaemia (Introna *et al.*, 1990). These results suggest that Myb in addition to promoting proliferation has a separable role in conferring a specific differentiation phenotype. Further evidence that Myb is necessary to promote granulocytic differentiation has came from antisense studies using HL60 cells. Treatment of these cells with antisense c-*myb* oligonucleotides prevents induced granulocytic differentiation; these cells, however, still have the capability to respond to monocytic inducers. This experiment suggests that these cells require 'c-Myb conditioned' proliferation to complete a granulocytic differentiation program (Ferrari *et al.*, 1992).

An obvious question is how can Myb confer a specific differentiation program during myelopoiesis when it is expressed throughout the haemopoietic system ? Clearly no protein acts in isolation and it may be that other factors are necessary which act in concert with Myb to determine a specific differentiation phenotype. There is emerging evidence of the co-operation of C/EBP proteins and Myb in determining the expression of myeloid-specific genes. It has been suggested that C/EBP factors are restricted to the myeloid compartment within haemopoietic tissues (Scott *et al.*, 1992; Katz *et al.*, 1993), and hence this is the only tissue in which both Myb and C/EBP factors are co-expressed, and together may determine a specific gene expression pattern in these cells (Ness *et al.*, 1993). The role of C/EBP in myelopoiesis is discussed in section 3.4. An intriguing question which remains to be answered is does Myb play a similar role in other lineages in conjunction with other as yet unidentified factors?

3.3 (v) B-Myb

It has been suggested that B-*myb* may functionally substitute for c-*myb* outside the haemopoietic system as it is ubiquitously expressed. Although it is possible that they have some overlapping functions, B-Myb has a number of

distinct properties. The human B-Myb been demonstrated to bind to a number of c-Myb binding sites *in vitro,* but can also bind to other sites which have little or no affinity for c-Myb (Mizuguchi *et al.*, 1990). These workers demonstrated that this protein could transactivate gene expression through these sites. In contrast the chicken and mouse B-Myb have been shown to act as repressors of transcription (Foos *et al.*, 1992; Watson *et al.*, 1993); the chicken B-Myb failed to transactivate the *mim-1* gene and the Mouse B-Myb failed to transactivate a Myb responsive promoter, and could repress c-Myb mediated transactivation of this construct. Fusion studies with the GAL4 DNA-binding domain and fragments of B-Myb failed to detect a transactivation domain. However the same study reported that both c-Myb and B-Myb could weakly transactivate the DNA polymerase α promoter (Watson *et al.*, 1993)

Antisense experiments with B-*myb* in HL60 and U937 cells resulted in inhibition of proliferation in the absence of differentiation, either spontaneous or induced (Arsura *et al.*, 1992). This is in contrast to the results obtained when HL60 cells were treated with antisense c-*myb* oligonucleotides. These cells retained the capacity for monocytic differentiation, suggesting these proteins have different roles.

3.4 The C/EBP family of transcription factors

The first member of the family to be identified was a heat stable DNA-binding protein present in rat liver nuclei (Johnson *et al.*, 1987). This protein was cloned and termed C/EBP (and subsequently C/EBPα) - CCAAT/enhancer binding protein due to its ability to bind both the CCAAT homology and the enhancer core homology (Landschulz *et al.*, 1988). A number of C/EBP family members have subsequently been cloned (Roman *et al.*, 1990; Descombes *et al.*, 1990; Chang *et al.*, 1990; Ron and Habener 1992), revealing a highly conserved leucine zipper and basic DNA-binding domain. This places the C/EBP family in the bZIP family

of transcription factors (other members include Fos, Jun, GCN4)

3.4 (i) DNA binding

A characteristic feature of the C/EBP family members is the degenerate nature of the sequences to which they bind. Although originally described as CCAAT binding protein, substitution of the first C for a G increases the affinity of C/EBP for this sequence ten-fold (McKnight and Tjian 1986). It has also been reported that C/EBPα can bind to a number of cAMP response elements (Bakker and Parker 1991), perhaps due to the similarity of the cAMP response element to the enhancer core homology. As these factors bind DNA as dimers and show conservation in the DNA binding domain, we would perhaps expect their binding sites to show dyad symmetry. However, although some sites show symmetry, many are asymmetric yet can still bind homodimers.

Comparison of C/EBPα binding to symmetric and asymmetric sites revealed that both sides of the asymmetric site made contacts which stabilised complex formation. This was despite only two of the seven nucleotides of the left matching those on the right. The contacts essential for C/EBP binding have two-fold symmetry and major groove contacts extend over a full turn of the helix for both asymmetric and symmetric sites (Nye and Graves 1990).

3.4 (ii) Functional domains

As mentioned previously the C/EBP family are bZIP transcription factors and this DNA-binding domain has been well studied. The transactivation domains have been mapped for C/EBPα. Two domains are required for the transactivation of the serum albumin gene, one of which is NH_2-terminal and the other centrally located. These transactivation domains do not show any homology to the acidic, proline or glutamine rich domains previously identified (Friedman and McKnight

1991). Another report identifies three functional domains in the NH_2-terminus, two transactivation domains sandwiching an 'attenuator' domain. This attenuator dampens down the transactivation mediated by these domains in a number of sequence contexts. Whether this domain serves to reduce the activity of these transactivation domains (which are extremely powerful in GAL4 fusion-reporter experiments) constitutively or can be regulated *in vivo* is unknown (Pei and Shih 1991).

3.4 (iii) The control of C/EBP activity

As many tissues co-express a number of family members, choice of dimerisation partner may have important functional consequences. As all cloned C/EBP factors contain a highly conserved leucine zipper this suggests that they be able to form heterodimers. Indeed, all family members tested have shown that they can dimerise in all intra-familiar combinations (Poli *et al.*, 1990; William *et al.*, 1991; Cao *et al.*, 1991). In most cases this heterodimerisation does not result in any apparent changes in DNA sequence specificity; however, dimers between C/EBPα and C/EBPγ have a higher affinity for the serum albumin promoter site D than either homodimer (Roman *et al.*, 1990). Another consequence of choice of dimerisation partner is differential transactivation properties. This was noted in a study with cotransfection with a synthetic C/EBP-responsive promoter and C/EBPα and C/EBPβ expression vectors. C/EBPα had higher constitutive activity than C/EBPβ. C/EBPα/β heterodimer had dimished constitutive activity, but C/EBPβ is IL-6 responsive and the heterodimer showed similar levels of induction as C/EBPβ/β homodimer (Poli *et al.*, 1990). This may function by altering the subcellular location of the heterodimer as C/EBPβ is largely cytoplasmic in PC12 cells but on stimulation with cAMP localises to the nucleus as well as showing increased DNA binding (Metz and Ziff 1991).

A common theme in transcriptional control is the presence of

heterodimerisation partners which are dominant negative regulators of transcription factor activity (c.f. section 2.3 (iii)). These transcription factors act by sequestering the positive activators into a heterodimer which fails to bind DNA (e.g. MyoD and Id). Examples of dominantly negative C/EBP family members have been isolated. CHOP is a developmentally regulated transcription factor which is present in a number of terminally differentiated cell types, and is capable of dimerising with C/EBPα and C/EBPβ. The basic region adjacent to the leucine zipper contains two prolines and heterodimers which include this transcription factor are unable to bind DNA *in vitro* (Ron and Habener 1992). Interestingly there are two proteins generated from the C/EBPβ gene, C/EBPβ and LIP. The two proteins are generated by different choice of translational start sites. LIP does not contain a transactivation domain and can repress C/EBPβ activity in a cotransfection assay. Interestingly these two isoforms are regulated during hepatic differentiation (Descombes and Schibler 1991), and have opposing effects as C/EBPβ (but not LIP) able to exert a growth arrest in hepatoma cells (Buck *et al.*, 1994).

Phosphorylation is an important mediator of transcription factor control, linking signal transduction pathways to gene regulation. The phosphorylation of the C/EBP family members has not been intensively studied, however some information is available. C/EBPα phosphorlation by PKC can reduce DNA binding *in vitro*, however whether this site has a role *in vivo* is not known (Mahoney *et al.*, 1992). The affinity of C/EBPβ and C/EBPδ for DNA can be reduced by PKA or PKC mediated phosphorylation at sites which are phophorylated *in vivo* (Trautwein *et al.*, 1994; Ray and Ray 1994)

C/EBPβ is phosphorylated in pituitary cells in response to increased intracelluar calcium concentrations as a consequence of activation of a calmodulin-dependent protein kinase. A reporter gene containing C/EBP binding sites was rendered calcium-responsive in these cells. The phosphorylation site is at a serine residue within the leucine zipper, suggesting that this may be important in the choice of dimerisation partner (Wegner *et al.*, 1992). Phosphorylation also mediates

the translocation to the nucleus of C/EBPβ in response to elevation of cAMP in PC12 cells, this translocation does not require protein synthesis and correlates with increased phoshorylation of C/EBPβ (Metz and Ziff 1991).

3.4 (iv) The role of C/EBP family in myeloid differentiation

Given the important role of the C/EBP family in differentiation in other systems, what role does this family play within the myeloid lineage? The expression pattern of the isoforms in myeloid cells differs from that of adipose and hepatic tissue, with an increase in C/EBPβ rather than C/EBPα as cells become terminally differentiated (Scott *et al.*, 1992). As discussed in section 3.1, the chick homologue of C/EBPβ (Katz *et al.*, 1993) and c-Myb are involved in the regulation of the *mim-1* gene (Ness *et al.*, 1993; Burk *et al.*, 1993). Surprisingly, when expressed in fibroblasts these transcription factors could activate the endogenous *mim-1* and the myeloid-specific lysozyme genes (Ness *et al.*, 1993). In the chick haemopoietic system, C/EBPβ is restricted to the myeloid compartment, and it has been suggested that together c-Myb and C/EBPβ may produce a myeloid-specific phenotype. The importance of C/EBPβ in myelopoiesis is underlined by its involvement in the regulation of the chick cMGF gene (Katz *et al.*, 1993; Sterneck *et al.*, 1992) which is distantly related to the mammalian G-CSF and IL-6 genes (Leutz *et al.*, 1984;1989). It is also of interest to note that those cells which v-Myb can transform express C/EBPβ.

Clearly this family of transcription factors plays a key role in myelopoiesis, which merits further study. The question of how close the mammalian system reflects the avian remains unanswered, as although the presence of C/EBP proteins have been reported to be limited to the myeloid compartment within the haemopoietic system (Scott *et al.*, 1992), the expression of C/EBPβ is much more widespread in mammalian tissues than in the chicken (Katz *et al.*, 1993).

3.5 The Ets family of transcription factors

The first member of the Ets family to be identified was v-*ets* in the chicken E26 transforming retrovirus as a fusion product with the Myb transcription factor (Nunn *et al.*, 1983). This Myb-Ets fusion is the result of an aberrant splicing event between the a cryptic splice donor site in c-*myb* exon VI and the splice acceptor site of exon I of the c-*ets-1* proto-oncogene (Leprince *et al.*, 1988). Since this gene was discovered, a large and expanding family of Ets proteins has emerged. Family members have been cloned from species as diverse as *Drosophila* to human and all members encode sequence-specific transcription factors.

3.5 (i) Functional domains of the Ets proteins

Sequence comparison of family members revealed a number of conserved regions, most notably the presence of a unique DNA binding domain. This domain has limited homology to the DNA binding domain of c-Myb, which has 9 tryptophan residues repeated at 18-19 amino acid intervals around a predicted α-helical structure (Karim *et al.*, 1990). The Ets domain contains a single triplet of tryptophan residues with 17-18 amino acid spacing. All family members identified todate contain this triplet, with the exception of Spi-1 (PU.1) and Spi-B which have replaced the third tryptophan with a tyrosine residue (Ray *et al.*, 1992).

The minimal DNA-binding domain of the chicken c-Ets-1 has been mapped by deletion analysis, this domain includes the Ets domain and surprisingly C-terminal sequences outside the Ets domain. These sequences may be important in mediating the specificity of DNA binding as these amino acids are conserved between Ets-1 and Ets-2, both of which can bind to the PEA3 motif with high affinity, whereas v-Ets which has lost these sequences cannot. However neither of these proteins can bind to the Ets binding site in the mb1 promoter with high affinity, both PU.I and v-Ets can. Deletion of these COOH terminal amino acids

increases the specific binding by 20 to 50 fold at this site (Hagman and Grosschedl 1992). However, a conflicting result (Lim *et al.*, 1992), demonstrated that c-Ets-1 was unable to bind a PEA3 site efficiently, but v-Ets could. Deletion of an inhibitory domain increased binding of c-Ets to this site.

Domain swap experiments involving p68 *c-ets-1* have identified three transactivation domains RI, II & III. When fused to a lexA DNA-binding domain both RI and RIII can transactivate, but RII has no activity alone. RII however has a negative effect on the transactivation by RI and a positive effect on that of RIII (Schneikert *et al.*, 1992). Interestingly the RII region of the protein maps to a sequence which has limited homology to the helix-loop-helix motif, and this region is conserved in a number of the Ets proteins (Pongubala *et al.*, 1992) although the functional significance of this homology is not clear. The transactivation domains of the PU.1 protein have also been mapped, and contain a glutamine rich and an acidic rich region which may be important in this activity (Pongubala *et al.*, 1992).

The Ets proteins also contain a nuclear localisation signal similar to that of the SV40 large T antigen, this region has been demonstrated to be sufficient to localise *c-ets-1* to the nucleus (McCleod *et al.*, 1992). A number of family members also contain a PEST sequence; these sequences are rich in proline, glutamine, serine and threonine and are thought to be a target for proteases. Cleavage at the PEST sequence could lead to the separation of the DNA-binding domain from the rest of the protein. In PU.I, the PEST sequence is involved in protein-protein interactions with other transcription factors and dimerisation may prevent access of proteases to this sequence which may have important regulatory consequences (Pongubala *et al.*, 1992).

3.5 (ii) The regulation of Ets protein activity

The control of Ets protein activity is mediated at a number of levels. Different Ets family members show very different expression patterns. For example

ets-1 is preferentially expressed in adult lymphoid tissues, with high levels also in the spleen and lung whereas expression of PU.1 is limited to macrophage and B cells. In contrast *ets-2* is expressed in almost every tissue (Seth *et al.*, 1990; Bhat *et al.*, 1987; Klemsz *et al.*, 1990). The expression can also be varied under different conditions; for example in T cells Ets-1 is at high levels in quiescent cells and decreases following T-cell activation, whereas Ets-2 levels follow the reciprocal pattern (Bhat *et al.*, 1990).

Tissue specificity is also generated by alternative splicing. Both chicken and human c-*ets-1* are alternatively spliced in a tissue-specific manner (LePrince *et al.*, 1988; Koizumi *et al.*, 1990); exons IV and VII, containing a negative regulatory region (which affects DNA binding), is spliced out in endothelial cells but not in T-cells. Interestingly Ets-2 also contains this domain but PU.I lacks it.

The Ets family members are also regulated post-translationally with phosphorylation being an important mediator of activity. Phosphorylaton of both Ets-1 and Ets-2 as a consequence of T-cell activity results in the loss of non-specific DNA binding activity of Ets-1 and stabilisation of Ets-2 half-life (Pognec *et al.*, 1988; Fujiwara *et al.*, 1988, 1990). TCF, an Ets family member is phosphorylated by MAP kinase in response to serum stimulation, which allows it to complex with the serum response factor over the serum response element (Gille *et al.*, 1992). Similarly activation of the oncogene-responsive element of the polyoma virus enhancer by TPA or non-nuclear oncogenes requires Raf-1 which is upstream of MAP kinase (Kryiakis *et al.*, 1992), which could phosphorylate Ets proteins to activate this element.

3.5 (iii) DNA binding

All of the Ets family of transcription factors bind to sites containing a core GGAA/T, however the surrounding sequence mediates the specificity of binding. Many of these Ets binding sites can be bound by a number of different Ets proteins;

for example the PEA3 element can be bound by Ets-1, Ets-2, Elk-1 and PEA3 and each of these proteins can transactivate DNA through this element (Wasylyk *et al.*, 1990; Rao and Reddy 1992; Xin *et al.*, 1992). *In vitro* the Ets proteins bind DNA in the absence of other proteins and appear to bind as monomers. *In vivo* the situation is more complex, with protein-protein interations playing a key role in mediating binding specificity and transactivation properties.

The oncogene-response region of the polyoma virus enhancer can be activated by TPA or non-nuclear oncogenes. This element contains a PEA3 site which can bind Ets-1 or Ets-2 in response to these signals. This PEA3 site is juxtaposed to an AP1 site and these show synergistic activity. Mutation in either of these sites reduces activity, but not to the level of only having a single element present suggesting that protein-protein interactions may involved in co-operative binding/transactivation (Wasylyk *et al.*, 1989; 1990). Adjacent AP1/PEA3 sites are found in several promoters of oncogene responsive genes e.g. collagenase, c-*fos* suggesting that this may be a common mechanism of activation. Another case of an Ets family member interacting with another protein allowing it to activate transcription is exemplified by the immunoglobulin K 3' enhancer. In this case PU.I recruits another factor to an adjacent site by protein-protein interactions (Pongubala *et al.*, 1993).

Two Ets family members SAP-1 and elk-1 exhibit the p62/TCF activity (Hipskind *et al.*, 1991; Dalton and Triesman 1992). This activity cannot recognise the serum response element (SRE) of the c-fos promoter alone. However, when the SRE is bound by the serum response factor it can bind to this complex and make sequence-specific contacts with the adjacent sequence (Rao and Reddy 1992). In the absence of any other proteins Elk-1 can also bind to the E74 sequence and act as a transcriptional activator. This illustrates the importance of the context of the Ets binding site and the interactions with other transcription factors in mediating DNA binding specificity and transcritpional activation.

3.5 (iv) Target genes for Ets family members

A large number of genes have been found to contain Ets binding sites in their promoters (Ho *et al.*, 1990) including oncogene responsive genes, a significant number of lymphoid-specific genes e.g. T-cell receptor α gene and genes involved in the degradation of the extracellular matrix e.g. stromelysin 1, collagenase (Wasylyk *et al.*, 1992) and genes involved in tissue-specific expression (Pahl *et al.*, 1993). A number of these genes have been demonstrated to be transactivated by a specific family member. These include PU.I, which has been demonstrated to be important in the myeloid-specific expression of the CD11b gene and Ets-1 which has been implicated in the megakaryocyte-specific expression (Lemarchandel *et al.*, 1993). Given the restricted expression pattern of these two genes, they may play an important role in the tissue-specific expression of a number of genes.

3.5 (v) The Ets proteins and cancer

The insertional activation of *fli-1* and *spi-1* in viral induced murine leukaemias and the role of the v-*ets* gene in conferring the ability to transform immature erythroid progenitors on the E26 virus is clear evidence of their importance in tumourigenesis. A number of common translocations found in human leukaemias are potentially involved in activating expression of a number of *ets* genes (reviewed in Papas *et al.*, 1990). The only conclusively demonstrated case of activation/alteration of *ets* genes playing a role in human tumours is in Ewings sarcoma. This is characterised by a translocation which results in a fusion protein containing the DNA binding domain of Fli-1 linked to a putative RNA binding protein, although the mechanism of this fusion proteins action is unknown (Delattre *et al.*, 1992)

3.6 Structure and expression of the human defensin genes

The chromosomal location of the human defensins has been mapped to 8p23 (Sparkes *et al.*, 1989). Fine mapping has not been undertaken for the human genes, however the rabbit genes have been studied in more detail. This has revealled the MCP1 and MCP2 genes are contained within a 13kb stretch of DNA (Lehrer *et al.*, 1989). The genes encoding HNP1 and HNP3 genes have been cloned and analysed, revealing that individuals contained different copy numbers of HNP1 and HNP3 encoding genes. The results from a limited family study suggested that individuals contain four defensin genes, two per chromosome 8, which are tightly linked (Lamb 1990). No information is available as to the copy number or chromosomal organisation of the gene encoding HNP4.

The expression of the defensin genes occurs predominantly in the myelocyte, with transcription coinciding with the production of primary granules. Consequently the defensin genes are expressed in both normal and leukaemic bone marrow, but only in the peripheral blood of chronic myeloid leukaemia patients and ocassionally in the peripheral blood of acute non-lymphocytic leukaemia. The expression of the defensin genes being coincident with the present of myelocytes in the samples (Wiedemann *et al.*, 1983; Daher *et al.*, 1988). Low levels of expression have also been reported in the promyelocyte cell line HL60, and levels are increased when the cells are induced to differentiate towards granulocytes (Daher *et al.*, 1988).

There have also been reports of defensin expression in cells other than immature granulocytes. In rabbit two defensins MCP 1 and MCP 2 are found in adult alveolar macrophage, and the levels of these two proteins can be increased by challenging the rabbit with Freunds adjuvant (Ganz *et al.*, 1989). A survey of tumour tissue and surrounding normal tissue revealed occasional defensin mRNA in lung and intestine (Daher *et al.*, 1988). Whether this is the result of infiltration of phagocytes stimulated to produce defensin mRNA is unknown.

3.7 Purpose of the project

As discussed in section 3, relatively few genes which are transcriptionally regulated during myelopoiesis have been analysed in detail. Similarly little information is available with regard to the transcription factors which control the differentiation program during myelopoiesis, or confer a myeloid phenotype.

Transcription factors have been demonstrated to play a central role in the control of differentiation in a number of systems, such as MyoD in myopoiesis (section 2.7). In addition to being key regulators of differentiation, the alteration of the normal properties of transcription factors is an important target in leukaemogenesis. This can be either a result of deregulated expression (for example as generated by the translocation activating c-*myc* in Burkitts lymphoma or TAL/SCL activation in T-cell ALL (Rabbitts and Boehm 1991; Baer 1993) or alteration of the properies of the transcription factor (most notably, as generated by the RARα gene rearrangements seen in APL, section 1.3 iv). As the alteration of the properties of transcription factors involved in normal proliferation/differentiation seems to be a key event in leukaemic transformation, the identiifcation of the transcription factors which control these processes is an important step in the understanding of how this process can be deregulated in leukaemia.

An approach to understanding the control of differentiation, is to analysis the regulation of genes which are expressed in a differentiation-specific manner. As described in section 3.6, the expression of the defensin genes is restricted to a very limited 'window' during myelopoiesis, namely the myelocyte. This allows a unique opportunity to study both how the differentiation stage-specific and myeloid-specific expression of these genes is mediated. Identification of the *cis*-acting sequences which are necessary for this control, and analysis of the transcription factors which mediate these activities will be instructive in understanding the control of normal myelopoiesis and how this may be altered to generate maturation arrest

and leukaemia.

The aims of this work were to identify those *cis*-acting sequences which control the differentiation stage-specific and myeloid-specific expression of these genes and to characterise and identify the *trans*-acting factors which bind these elements.

Part 2: Materials and Methods

Chapter 4: Methods

4.1 Cell culture

4.1 (i) Cell culture conditions

Cells were obtained from Beatson Institute stocks, which were regularly tested for the presence of mycoplasma using the Hoechst 33258 staining method (Chen 1977).

Most cells were maintained in special liquid medium (Gibco BRL) supplemented with 10% Foetal calf serum and 2mM glutamine, gassed to 5% CO_2 and incubated at 37°C. Where appropriate, cells were maintained in RPMI-1640 (Northumbria Biologicals Ltd), supplemented with 0.2% sodium bicarbonate, 2mM sodium pyruvate, and 10% foetal calf serum.

Cells cultures were routinely passaged every 2-3 days to maintain cells in a continuously growing state. Monitoring of cell numbers was carried out using a Coulter counter (Coulter Electronics Ltd., Bedfordshire UK) or a haemocytometer. Frozen stocks were stored in liquid nitrogen in 10% DMSO.

4.1 (ii) Induction of differentiation

The exact protocol depended on the cell line to be induced and the agent to be used. Typically the cells were split the day prior to the induction. If the agent prevented further cell division i.e. TPA treatment of HL60 cells, then the cells were split to $0.5x10^6$/ml on the previous day. If the agent did not block cell division then consideration of the number of divisions that would take place during the experiment was necessary.

Dimethyl sulphoxide; HL60 cells were treated with DMSO to give a final concentration of 1.5% (v/v) in the medium.

Retinoic acid; Stock solution was made up fresh for each induction at 10^{-2} M, and stored at -20°C in the dark. HL60 cells were treated with retinoic acid to give a final

concentration of 10^{-6} M. This was added fresh every day of the induction period. The induction was carried out in the dark.

TPA; HL60 cells were treated with TPA (dissolved in acetone) to give a final concentration of $1.6x10^{-7}$M.

4.1 (iii) Cell staining - May-Grunwald and Giemsa staining

Cells were spun onto glass slides using a Shannon cytocentrifuge at 2000g for 5 minutes and air dried. The cells were then fixed in 70% methanol. May-Grunwald stain was prepared by diluting 2 volumes of stain in 3 volumes of Sorensen's buffer pH 6.85 (0.066M disodium orthophosphate, 0.066M potassium dihydrogen orthophosphate) and filtering through Whatman 3MM paper. Giemsa stain was also diluted in Sorensen's buffer (1 volume stain: 9 volumes buffer) and filtered. The slides were stained for 5 minutes in May-Grunwald and then for 10 minutes in Giemsa. They were then washed in double-distilled water, air dried mounted in DPX mountant (BDH), and examined by light microscopy.

4.1 (iv) Nitroblue tetrazolium

$1x10^6$ cells were harvested by centrifugation in an MSE Centaur bench centrifuge at 2000g for 5 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in 10mM tetradecanoyl phorbol acetate (Sigma) containing 0.2% (w/v) nitroblue tetrazolium in PBS. The cell suspension was incubated at 37°C for 25 minutes. 0.5ml of the cell suspension was removed and the cells centrifuged onto a clean glass slide using a Shannon cytocentifuge. The slide was then stained as described in the previous section.

4.1 (v) Preparation of white blood cells from buffy coats

Buffy coats were supplied by the West of Scotland Blood Transfusion Service. To the buffy coat, 5 volumes of erythrocyte lysis buffer (0.83% NH_4Cl (w/v), 0.037g/l NA_2EDTA, 1g/l $KHCO_3$) was added and incubated on ice for 10

minutes. This was then centrifuged 1500g/5 minutes/4°C in a Beckman J6B, the supernatant was removed and the pellet was resuspended in PBS. The procedure was repeated until all the red colouring in the pellet was lost. The pellet was then washed in PBS.

4.2 Recombinant DNA techniques

4.2 (i) Host strains

E.coli. XL1 (Bullock *et al.*, 1987) were obtained from Institute stocks, and were grown in L-broth at 37°C with good aeration, ampicillin (100µg/ml) and tetracyclin (12.5µg/ml) were used when appropriate.

4.2 (ii) Generation of competent cells

4ml of an overnight culture of XL1 was inoculated in 500ml of L-broth and grown at 37°C with good aeration until the absorbance at 550nm had reached between 0.4 and 0.6 (1cm light path, L-broth blank). The cells were collected by centrifugation in a Beckman J6B at 3000g/10 minutes/4°C. The pellet was resuspended in 250ml 100mM $MgCl_2$ and repelleted. The supernatant was discarded and the pellet resuspended in 250ml of 100mM $CaCl_2$, incubated on ice for 40 minutes and respun. The pellet was resuspended in 15ml of 100mM $CaCl_2$/20% glycerol. Aliquots were flash frozen in liquid nitrogen and stored at -70°C.

4.2 (iii) Transformation of bacterial cells

Competent cells were thawed slowly on ice, and 100µl placed into pre-chilled 15ml falcon 2059 tubes. Plasmid or ligation mix was pipetted into the cells, mixed by gently tapping the tube and incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 2 minutes. 1ml of L-broth was then added and incubated for 1hr at 37°C in a New Brunswick G25 shaker at 225rpm. 100-200µl was then spread onto 1.5% (w/v) agar L-broth plates supplemented with the

appropriate antibiotic. The plates were then allowed to dry and then inverted and placed in a 37°C incubator overnight.

4.2 (iv) Glycerol stocks

E.coli. host strains or hosts bearing useful plasmids were stored as glycerol stocks for future retrieval. Stationary cultures in L-broth were mixed with an equal volume of glycerol and stored at -20°C.

4.3 Preparation of plasmid DNA

4.3 (i) Minipreparation of plasmid DNA

Colonies were picked from agar plates using a sterile toothpick and used to inoculate 10ml of L-broth (with appropriate antibiotics), and incubated overnight at 37°C with good aeration. Cells from 1.5ml of this culture were pelleted in a microfuge, and resuspended in 100µl of solution A (50mM glucose, 25mM tris.Cl pH 8, 10mM EDTA, 4mg/ml lysozyme) and stored on ice for 5 minutes. 200µl of alkaline lysis buffer (1% SDS, 0.2M NaOH) was added, mixed gently and incubated on ice for 5 minutes. 150µl of 3M NaAc (pH 5.2) was added, the solution was vortexed and microfuged at 12000g/5minutes/4°C. 380µl of the supernatant was removed and a phenol/chloroform extraction was performed. Plasmid was then precipitated by the addition of 800µl of ethanol, incubated at room temperature for 2 minutes, then pelleted in a microfuge at 12000g/10 minutes/4°C. The pellet was resuspended in 50µl of TE containing 10µg/ml of RNAseA.

4.3 (ii) Large scale plasmid preparation

1ml of an overnight culture was inoculated into 250ml of LB medium containing the appropriate antibiotics and grown overnight in an orbital shaker at 37°C. The culture was harvested by centrifugation in a Beckmann J6B at 3000g/10

minutes/4°C. The supernatant was discarded and a Quiagen (Quiagen Inc.) large scale plasmid preparation was carried out as recommended by the manufacturer.

4.4 Quantification of nucleic acids

Nucleic acids were quantified by measuring absorbance at 260nm in a Beckman DU 650 spectrophotometer.

4.5 Restriction digests and gel electrophoresis

4.5 (i) Restriction digests

For plasmid DNA, typically 5 units of enzyme/µg of DNA was added in the appropriate buffer and incubated for 1hour. Restriction digestion of genomic DNA was carried out using the desired enzyme at a concentration of 10 units of enzyme/µg of DNA and incubated for 12 hours. Spermidine pH 7 was included to a final concentration of 3mM. At the completion of the digest, the reaction was stopped by the addition of EDTA pH 8 to 20mM.

4.5 (ii) Agarose gel electrophoresis

An agarose solution was made using 1xTBE buffer (89mM Tris base, 89mM boric acid, 2mM EDTA, pH 8.0) and an appropriate amount of agarose (dependent on the size of the DNA molecules to be separated, see Sambrook *et al.*, 1989). This was heated in a microwave and then ethidium bromide was added to a final concentration of 0.5µg/ml and the solution was poured into a horizontal gel mould when hand hot. The DNA samples were mixed with one-tenth volume of gel loading buffer (50% glycerol, 1% bromophenol blue, 10mM sodium phosphate pH 7) and applied to the wells using a micropipette. Gels were run at 10-100 volts depending on the size of the gel tank and the time available. Markers were run simultaneously and were usually lambda 'phage DNA digested with *Hind*III for the sizing of large fragments and θX174 DNA digested with *Hae*III for sizing of small fragments (Promega). The DNA was then visualised by UV fluorescence on a

Chromato-Vue transilluminator, then photographed through a red number 9 Kodak Wratten gelatin filter.

4.5 (iii) Non-denaturing polyacrylamide gel electrophoresis (PAGE)

A 30% stock acrylamide solution was made up containing 1%N,N'methylbisacrylamide. This stock solution was used to cast vertical gels of various concentrations, depending on the size of the fragments to be resolved. The gel contained TBE, the concentration of which was varied depending on the concentration of acrylamide used in the gel (see Sambrook *et al.*, 1989 for details). The gel was set by the addition of 250µl of 10% ammonium persulphate (APS) and 100µl of TEMED per 50mls of gel solution. The gel was run in a vertical tank at a constant current of 25mA, and the DNA was visualised by staining with a solution of 0.5µg/ml ethidium bromide and UV illumination.

4.5 (iv) Denaturing polyacrylamide gel electrophoresis

These gels were cast on glass plates (20cm x 45 cm) which had been cleaned and siliconised. The plates were separated by 0.5mm spacers and the polyacrylamide gels were made up as described by Sambrook *et al.*, (1989); 6% acrylamide, 0.2% bis-acrylamide, 1 X TBE, 8M urea, 0.08% APS and 0.05% TEMED. Running conditions were usually 1600V, 50W. At the end of the run, the glass plates were separated and a sheet of Whatman 3MM paper was placed on top of the gel. The gel sticks to the Whatman paper and can be peeled from the plate. It was covered with Saran-wrap, dried on a commercial gel drier at 80°C for 2 hours before autoradiography.

4.5 (v) Purification of DNA fragments from agarose gels

The fragment was excised from the gel using a scalpel. This gel fragment was placed into a pre-prepared eppendorf column and microfuged at 5000g for 5 minutes. The resultant liquid was subject to phenol/chloroform extractions,

precipitated and resuspended in an appropriate volume. The columns were prepared by placing a 0.5ml eppendorf tube inside a 1.5ml screw cap eppendorf tube. A hole was pierced in the base of the 0.5ml tube and this was filled with polyallomer wool. The agarose fragment was placed on top of the wool. The liquid is recovered after centrifugation and is found in the bottom of the screw cap eppendorf tube.

4.6 Subcloning of DNA

4.6 (i) Generation of vectors for cloning fragments

Plasmids were digested with the appropriate restriction enzyme, included in the reaction was 1µl of calf intestinal alkaline phosphatase (CIAP, Boehringer Mannheim). For the generation of blunt-ended vectors, the reaction was transferred to a 56°C water bath and incubated for a further 30 minutes with the addition of another 1µl of CIAP. EDTA (pH8) and SDS were added to a final concentration of 5mM and 0.5% respectively. Proteinase K was added to a concentration of 100µg/ml and the reaction was incubated at 56°C for 30 minutes. The reaction was phenol/chloroform extracted, chloroform extracted and precipitated, the pellet was then resuspended in 100µl of H_2O.

4.6 (ii) Preparation of inserts for cloning

Inserts were generated by either restriction enzyme digest of plasmid DNA or by PCR followed by digestion. These reactions were phenol/chloroform extracted, chloroform extracted and purified after agarose gel electrophoresis. The inserts were quantified after purification, by gel electrophoresis of a fraction of the insert and comparison with markerDNA of known concentration.

4.6 (iii) Ligation reactions

For each ligation the following controls were set up; vector alone, unphosphorylated vector and insert alone. To each reaction 100ng of vector was added, and usually a four-fold molar excess of insert. In addition, another three

reactions were set up containing different amounts of insert, ranging from equimolar to a 10 fold molar excess of insert to vector. Water was then added to a final volume of 7µl. The reaction was heated to 45°C for 5minutes and then chilled on ice for 5 minutes. To each reaction, 2µl of 5X T4 ligase buffer (supplied with the enzyme) and 1µl of enzyme (NBL) was added. For 'sticky end' ligations, 0.1 Weiss units was added, whereas 0.5 Weiss units were added for blunt end ligations. The reaction was incubated at 14°C for 4-16 hours, and typically 2µl of the ligation mixture was used for transformation.

4.7 Synthesis of oligonucleotides.

Oligonucleotides were synthesized at the Beatson Institute on an Applied Biosystems model 381A DNA synthesizer according to the manufacturers instructions. 5' trityl groups were removed by the machine, and the DNA immobilized on a column. The DNA was eluted from the column in 29% (v/v) ammonia, by passing the ammonia continuously through the column for three 5-minute periods separated by 20 minute intervals. The resulting DNA-ammonia solution was sealed in a glass vial, incubated overnight at 55°C, then precipitated after additon of ammonium acetate to 0.3M with 3 volumes of ethanol. DNA was pelleted by centrifugation at 12000g in a Sorvall SS-34 rotor for 15 minutes, redissolved in 3 ml of aqueous 0.2 M sodium acetate, precipitated with 3 volumes of 100% ethanol, then pelleted by centrifugation. The DNA pellet was washed with 70% (v/v) ethanol and dissolved in 1 ml of distilled water. After quantitation by spectrophotometry, oligonucleotides were stored at -20°C until required.

4.8 Polymerase chain reaction

4.8 (i) Design of PCR oligonucleotides.

In each case, two single-stranded oligonucleotide primers generally 20-40 bases in length, were designed such that they were complementary to opposite strands and opposite ends of the DNA sequence of interest. Oligonucleotides

contained a number of nonspecific nucleotides at their 5' ends followed by a restriction enzyme recognition sequence, allowing subcloning of the PCR-generated DNA fragment into the appropriate restriction sites of the chosen plasmid vector. The 3' end of oligos was either a G or a C base if possible, in order to enhance priming from the oligonucleotide.

4.8 (ii) PCR reactions.

The template for PCR reactions was linearized plasmid DNA, containing the sequence of interest at a concentration of 100ng/reaction, was combined in a PCR reaction tube with 25 pmols. of each PCR primer, 10µl of Taq polymerase buffer (provided with Taq enzyme by the manufacturers), 2µl from 5 mM stock solutions (in distilled water) of each of dATP, dTTP, dGTP and dCTP, 6µl of 25mM $MgCl_2$, 0.5µl of Taq polymerase and distilled water to a final volume of 100µl. The solution was overlayed with 50µl of parafin oil, then incubated in a PCR machine (Perkins-Elmer) for 25 of the following cycles: 1) 95°C for 1 minute; 2) 55°C for 1 minute; 3) 72°C for 90 seconds. Reactions were incubated for 15 minutes at 72°C after the final cycle, then gradually cooled to 4°C. Each reaction was extracted with one volume of chloroform, ethanol precipitated, analysed by agarose gel electrophoresis and the relevant band purified, restriction digested and resuspended in distilled water.

4.9 Sequencing plasmid DNA

4µg of plasmid in a total volume of 20µl of 0.2M NaOH was incubated at 37°C for 30 minutes. The DNA was then precipitated by the addition of 8µl 5M ammonium acetate pH 5.2 and 100µl ethanol and incubated for 5 minutess on dry ice, then spun at 12000g/5 minutes/4°C in microfuge. The pellet was washed in 70% ethanol and resuspended in 7µl H_2O 1µl of primer (3ng), 2µl reaction buffer (USB sequenase kit) and incubated at 65°C for 2 minutes then slowly cooled to

room temperature. The sequencing reactions were then carried out using a sequenase kit (USB) as recommended by the manufacturers.

4.10 Isolation of RNA

Preparation of total cytoplasmic RNA was prepared using the RNAzol B method (Biogenesis Ltd.). Cells were harvested in an MSE bench top centrifuge at 1500g for 5 minutes. The cells were resuspended in RNAzol at 2ml per 10^7 cells and 200µl of chloroform. The samples were vortexed for 15 seconds, and incubated on ice for 5 minutes. The samples were then centrifuged at 12000g at 4°C for 15 minutes. The upper aqueous phase was transferred to a fresh tube, precipitated with a half volume of isopropanol at 4°C for 15 minutes and centrifuged at 12000g for 15 minutes at 4°C. The pellet was washed with 70% ethanol and resuspended in Diethylpyrocarbonate (DEPC) treated water.

4.11 Northern blot analysis

4.11 (i) Agarose electrophoretic separation of RNA

To 87ml of DEPC treated H_20, 1g of agarose and 10ml 10xMOPS (3-(N-morpholino)propanesulphonic acid, 50mM sodium acetate, 10mM EDTA pH 7) was added. This was dissolved by heating in a microwave, cooled to hand hot and then 5.1ml formaldehyde solution was added. The gel was then cast and run in 1xMOPS buffer. Samples of RNA were dissolved in DEPC treated water and 10µl sample buffer (which was made up fresh before use; 0.75ml deionised formamide, 0.15ml 10x MOPS, 0.24ml formaldehyde 0.1ml H_2O, 0.1ml glycerol, 0.08% bromophenol blue) was added per 2µl of sample prior to electrophoresis. An RNA ladder (BRL) was run to provide markers on each gel.

4.11 (ii) Northern transfer of RNA

After electrophoresis the gel was soaked in distilled H_2O for 1 hour. The blot was set up as described in Sambrook *et al.*, (1989), and the RNA was

transferred onto Hybond N^+ nylon membrane (Amersham International) in 10xSSC overnight by capillary action. The RNA was fixed to the membrane using a UV Stratagene 1800 crosslinker.

4.12 Southern blot analysis

After electrophoretic separation of the digested DNA samples, the gel was soaked in 1.5M NaCl, 0.5M NaOH for 2x 20 minutes with gentle shaking, followed by soaking in 1M ammonium acetate, 0.02M NaOH for 2x 30 minutes. The blot was then set up as described in Sambrook *et al.*, (1989) and transferred to Hybond N^+ nylon membrane in 1M ammonium acetate, 0.02M NaOH overnight. The filter was then rinsed in 2xSSC and the DNA was crosslinked using a UV Stratagene 1800 crosslinker.

4.13 Random-primed radiolabelling of DNA probes.

All DNA probes used for hybridisation to Northern and Southern blots were labelled for 30 minutes at 37°C with [α-^{32}P]-dCTP, using a random-priming kit as recommended by the suppliers (Boehringer-Mannheim). After the labelling reaction, the probe was separated from unincorporated nucleotides on a Nick-column (Pharmacia) equilibriated with 0.1x SSC, 0.1% (w/v) SDS. Radiolabelled probes were denatured by boiling for 5 minutes prior to use.

4.14 Hybridisation conditions

Hybridisations were carried out as recommended by the manufacturers of Hybond N^+ (Amersham International), with the inclusion of 50% formamide for Northern blots which were carried out at 42°C whereas Southern blots were hybridised in the absence of formamide at 65°C. Pre-hybridisations were carried out for a minimum of 4 hours, and fresh hybridisation buffer was added containing denatured radiolabelled probe at 10^6CPM/ml and incubated for 16 hours. The filters were then washed as recommended by the manufacturers.

4.15 DNAse1 hypersensitivity analysis of chromatin structure

4.15 (i) Isolation of nuclei

The amount of starting material varied, but $1x10^8$ cells was optimal. The cells were harvested by centrifugation in a Beckman J-6B at 4000g/10 minutes/4°C, washed in PBS and re-centrifuged . The pellet was washed in 100ml TMS (0.25M sucrose, 10mM Tris, 5mM $MgCl_2$, 7mM β-mercaptoethanol) and respun, the resultant pellet was resuspended in TMS/0.25% triton and repelleted at 4000g/10 minutes/4°C. The pellet was washed in 100ml TMS X 2 and resuspended in 5ml TMS (the integrity of nuclei was assessed by light microscopy).

4.15 (ii) DNAse1 treatment and purification of DNA

The nuclei were split into a number of tubes each containing 0.5-1ml nuclei, to each of these tubes was added 1µl 1M $MgCl_2$ and DNAse1 (Lorne Laboratories Ltd) between 0.1 and 10µg/ml final concentration. The samples were made up to 1ml with TMS and incubated at 37°C for 5 minutes. The reactions were then stopped by the addition of 9ml of stop solution (10mM Tris pH7.8, 0.5% SDS, 5mM EDTA, 50µg/ml proteinase K). These reactions were then incubated at 37°C overnight with gentle agitation. The solution was then extracted sequentially, twice with 1 volume of phenol, once with 2 volumes of phenol/chloroform/IAA, and once with 1 volume of chloroform/IAA. Sodium chloride was added to a concentration of 0.2M, nucleic acids were ethanol precipitated overnight at -20°C, pelleted, dissolved in 2 ml of 5x TE [pH 8.0] buffer, and digested overnight at 4°C with 100µg/ml of DNase-free RNaseA. Samples were then sequentially extracted with phenol/chloroform/IAA and chloroform/IAA, the nucleic acids were ethanol precipitated overnight, pelleted and resuspended in TE buffer.

4.16 Nuclear protein preparations

4.16 (i) Protease inhibitors

All solutions used for the preparation of nuclear protein contained a cocktail of the following protease/phosphatase inhibitors which were added fresh to the solutions: 50mM phenylmethyl-sulphonyl fluoride made up in isopropanol (100x); 50mM benzamidine (100x); 1M sodium butyrate (100x); 200mM levamisole (100x); 100mM sodium orthovanadate (2000x); leupeptin 1mg/ml (1000x); aprotinin 1mg/ml (1000x); bestatin 1mg/ml (1000x); pepstatin A 1mg/ml in ethanol (1000x); 14.4M β-mercaptoethanol (2000x). All inhibitors were obtained from Sigma.

4.16 (ii) Minipreparation of nuclear extracts

Typically $1x10^7$ cells were used as starting material, although as few as $5x10^5$ cells could be used. All centrifugations of less than 30 seconds were carried out at room temperature in a microfuge; between the steps samples were placed on ice. Adherent cells were harvested by scraping into 1.5ml of ice cold PBS, suspension cells were pelleted and resuspended in 1.5ml PBS. Cells were microfuged for 10 seconds and resuspended in 400µl buffer A (10mM Hepes-KOH pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT) by flicking the tube. The cells were allowed to swell on ice for 10 minutes, and then vortexed for 10 seconds. Samples were then microfuged for 10 seconds and the pellet was resuspended in 100µl of buffer B (20mM Hepes-KOH pH7.9, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT) and incubated on ice for 20 minutes. Cellular debris is removed by centrifugation 12000g/2 minutes/4°C and the supernatant is aliquoted and stored at -70°C. The yield was typically 50-75µg/10^6 cells (Andrews and Faller 1991)

4.16 (iii) Large scale preparation

This preparation was typically carried out using a minimum of $5x10^8$ cells. Cells were harvested by centrifugation in a Beckman J-6B at 4000g/5 minutes/4°C. The pellets were washed in PBS and respun. The pellets were pooled in a falcon tube in 50ml of PBS and respun at 4000g/15 minutes/4°C. The pellets were then washed in TMS and spun at 4000g/10 minutes/4°C. The supernatant was discarded and the packed cell volume was estimated. The pellet was then resuspended in 4x this volume of TMS/0.1% triton and incubated on ice for 10 minutes. This was then homogenised using Dounce B homogeniser with 25 strokes. This solution was placed in falcon 2059 tubes and spun in a Sorvall HB-4 rotor at 3500g/10 minutes/4°C. The pellet was resuspended in TMS/0.1% triton and respun. The pellet was then washed in TMS and repelleted. The pellet was then resuspended in TMS to a volume of 10ml. A total of 750µl of 5M NaCl was added slowly to nuclei stirring on ice. The suspension was incubated on ice for 30 minutes. This was then spun at 3500g/10 minutes/4°C in a Sorvall HB-4 rotor and the supernatant was stored on ice. The pellet was resuspended in 5ml TMS 375µl of 5M NaCl and stirred on ice for 15 minutes. This was then pelleted at 3500g/10 minutes/4°C and the two supernatants were pooled and spun at 100 000g in an ultracentrifuge for 60 minutes at 4°C. The volume of the supernatant was accurately estimated and 0.4533g of ammonium sulphate was added per ml of supernatant and the suspension was stirred on ice for 30 minutes. The precipitate was spun in a Sorvall HB-4 rotor at 12000g/15 minutes/4°C, and the pellet was resuspended in TMS/0.35M NaCl and dialysed over night against 1 litre storage buffer (20mM Hepes pH 7.8, 50mM NaCl, 5mM $MgCl_2$, 0.1mM EGTA, 5mM β-mercaptoethanol, 20% glycerol). The extract was then aliquoted and stored at -70°C.

4.17 Electrophoretic mobility shift assays

4.17 (i) Preparation of double stranded probes

10µg of complementary oligonucleotides were mixed in 100µl of TE. This was heated to 90°C and slowly cooled to room temperature. Oligonucleotides were labelled by one of two methods; oligonucleotides which were blunt ended were labelled with γATP using T4 polynucleotide kinase (NBL), those with a nucleotide overhang were labelled using klenow polymerase (NBL) with the appropriate radiolabelled dNTP(s). Reactions were performed as described in Sambrook *et al.*, (1989). Labelled oligonucleotides were purified after separation by PAGE. The labelled oligonucleotide was visualised by autoradiography, the band was cut out and eluted into 1ml of TE by incubation at 37°C for 12 hours.

4.17 (ii) Binding reaction

The appropriate volume of nuclear extract (typically containing 10µg) was incubated along with 6µg poly (dI.dC), 5µl gel shift buffer (final concentration 10mM Hepes pH8, 0.5mM EDTA, 100mM NaCl, 10mM $MgCl_2$, 1mM DTT, 10% glycerol, 0.1mg BSA), approximately 500pg labelled oligonucleotide probe and H_2O to a total volume of 25µl. Reactions were incubated at room temperature for 20 minutes before being loaded onto the gel. Where necessary, cold competitor was added prior to the addition of labelled probe and incubated on ice for 10 minutes. Samples were electrophoresed through 0.5xTBE, 6% acrylamide gelss in 0.5xTBE running buffer (pre-run for 1hr at 25mA) for approximately 3 hours at 25mA; the gel was then dried prior to autoradiography.

4.18 *In vitro* transcription and translation

The sequence to be transcribed was within the Bluescript plasmid, such that the T7 promoter was 5' of the gene. 10µg of the plasmid was linearised by restriction digestion of a unique 3' site, phenol/chloroform and chloroform extracted, precipitated and resuspended at in 20µl of TE buffer. *In vitro*

transcription was carried out with 1µg of plasmid in 1x transcription buffer (40mM Tris (pH 7.5), 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl) with 2.5mM NTPs, 10mM DTT, 40U of RNasin and 30U of T7 RNA polymerase in a final volume of 20µl for 90 minutes at 37°C. 2µl of this reaction was then used to prime a rabbit reticulocyte translation reaction (Promega), which was carried out according to the manufacturers instructions. Aliquots from this reaction were then used in EMSA reactions.

4.19 DNAse1 footprinting

4.19 (i) Generation of radiolabelled probes.

The region of to be footprinted was subcloned into bluescript. 10µg of DNA was cut with a restriction enzyme linearising the plasmid at one end of the sequence to be footprinted. After completion of the digest this reaction was phenol/chloroform extracted and precipitated. The resulting pellet was then resuspended in nick translation buffer (Sambrook *et al.*, 1989) and the fragment was end labelled with the appropriate radionucleotide (e.g. *Hind*III requires radioactive dATP as the first nucleotide to be 'filled in' is dATP) as described in section 4.15 (i). Unincorporated nucleotide was removed by precipitation. The pellet was then resuspended in H_2O and digested with a restriction enzyme which cleaves the DNA at a second site which marks the end of the probe. These fragments were separated by agarose electrophoresis and the band of interest was visualised by autoradiography, purified and resuspended in H_20.

4.19 (ii) Footprint analysis

Each reaction contained 1µl of probe (~20000 CPM), 6µg of poly (dI.pdC) (Sigma), nuclear protein and storage buffer (50mM NaCl, 20mM Hepes (pH7.9), 5mM $MgCl_2$, 0.1mM EDTA, 1mM DTT, 20% glycerol) to 100 µl. The reactions were incubated at 4°C for 1hour and at room temperature for 20 minutes. In addition control reactions containing an equal amount of BSA instead of nuclear

protein were included in each experiment. Reactions were set up in triplicate and a range of DNAse1 treatments were given to test which was optimal. 1-10µl of a 1/10 dilution of HPLC purified RNAse free DNAse1 (2mg/ml stock, Worthington Ltd.) was added to the reactions which were incubated for 20 seconds at room temperature before the addition of 100µl of stop solution made up fresh before use (1.5ml= 1.3ml 100mM Tris (pH 8) 10mM EDTA, 70µl 10% SDS, 60µl proteinase K (10mg/ml), 38µl 4M NaCl). These reactions were then incubated at 30°C for 30 minutes and then 90°C for 3 minutes. The reactions were 2x phenol/chloroform and chloroform extracted, then precipitated by the addition of 10µl of 5M LiCl, 600µl of ethanol and incubation in dry ice for 15 minutes. The DNA was pelleted in a microfuge at 12000g/4°C for 10 minutes. The pellet was washed in ice cold 95% ethanol and air dried. This was then redissolved in 10µl of stop buffer (95% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue). The samples were heated at 90°C for 3 minutes and frozen at -70°C. The samples could then be thawed at leisure and ran on a denaturing polyacrylamide gel (minimum of 5000 CPM/lane).

4.19 (iii) Maxam and Gilbert sequencing reactions

Footprinting probes were sequenced using a kit from NEN according to the manufacturers instructions.

4.20 Transfection into mammalian cell lines

4.20 (i) Electroporation

The cell cultures were passaged the day prior to the transfection so the cultures were exponentially growing at the time of electroporation. Cells were harvested in a MSE bench top centrifuge at 1800g for 5 minutes ($1x10^7$ cells were used per transfection) the medium was removed and stored at 37°C. The cell pellet was resuspended in 0.2ml fresh medium and the cell suspension was added to a

cuvette (BioRad) containing DNA in 50µl of TE. The cells were electroporated in a BioRad Gene Pulser at 250V, 960µF. 1ml of fresh medium was added to each cuvette and the cells were placed in tissue culture flasks containing 4ml of conditioned medium with penicillin and streptomycin. The cells were grown in a humid 5% CO_2 incubator at 37°C and then medium was removed 18 hours after transfection for analysis.

4.20 (ii) Assay for secreted placental alkaline phosphatase (SPAP).

200µl of cell growth medium was removed from transfected cells for use in the SPAP assay (Henthorn *et al*, 1988). Cell growth medium contains high levels of phosphatase activity, which is sensitive to heat-treatment; in contrast, SPAP is highly resistant to heat-treatment (Henthorn *et al*, 1988). Hence, cell growth medium from transfected cells was incubated at 65°C for 2 hours prior to use, then briefly microfuged, and 100µl aliquots transferred to plastic cuvettes. DEA buffer (1M diethanolamine, 0.28M NaCl, 0.5 mM $MgCl_2$, pH 9.85), and a 100mM stock of p-nitrophenylphosphate (PNPP) in DEA buffer, were used immediately prior to the assay, to make a 5 mM stock of PNPP in DEA buffer. 1 ml of this solution was pipetted onto each heat-treated sample. Phosphatase treatment of PNPP produces p-nitrophenol, which has a yellow colour; thus reactions were incubated at 37°C until yellow colour was seen to develop. Colour development was assayed spectrophotometrically, by measuring absorbance at 405nm, using heat-treated medium from mock-transfected cells as a blank.

4.20 (iii) Assay for human growth hormone (hGH).

This assay is based on the work of Selden *et al* (1986) and uses the Allegro dual growth hormone-specific monoclonal antibody system as recommended by the supplier (Nichols Institute Diagnostics Ltd.). Briefly, hGH secreted by transfected cells into the cell growth medium is recognised by two anti-hGH protein murine monoclonal antibodies, to different hGH protein epitopes, one of which is [^{125}-I]-

labelled and the other of which is biotinylated and linked to avidin-coated glass beads. hGH present in the cell growth medium becomes sandwiched between the two antibodies, thus allowing linkage of [^{125}-I] with the glass beads in amounts proportional to the levels of hGH present in the sample of cell growth medium. The level of [^{125}-I] thus linked to the glass beads is monitored by its gamma-particle emission, using a Beckmann gamma-counter. 200µl samples of medium were removed from transiently transfected cells, and 100µl of each sample was assayed for human growth hormone (hGH) activity.

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

4.21 Western blotting

4.21 (i) Preparation and quantification of samples

Samples were prepared by washing cells in PBS and then resuspending in sample buffer (0.0625M Tris pH 6.8, 2% SDS, 10% glycerol, 5%

mercaptoethanol). The samples were then boiled for 5 minutes and sonicated. Protein content was quantified using a BioRad protein assay kit as recommended by the manufacturer.

4.21 (ii) SDS-PAGE

Samples were electrophoresised in vertical polyacrylamide gels. These gels were composed of a stacking gel [20ml=3ml acrylamide stock (28.2% acylamide:0.8%bisacrylamide), 5ml buffer (0.5M Tris pH 6.8, 0.4% SDS), 14ml H_2O, 60µl 10% APS, 20µl TEMED] and a running gel [40ml=13ml acrylamide stock, 9ml buffer (1.5M Tris pH8.8, 0.4% SDS), 200µl APS, 20µl TEMED, H_2O to 40ml]. The running buffer was 25mM Tris pH 8.3, 192mM glycine, 0.1% SDS. Samples were ran along with pre-stained size markers.

4.21 (iii) Western blotting

After electrophoretic separation proteins were blotted onto nitrocellulose membranes (Schleicher and Schull BA85) using a Sartorious semi-dry blotting apparatus as follows. Twelve pieces of Whatman 3MM paper and one piece of membrane were cut to the size of the gel and equilibrated in transfer buffer (60mM Tris, 50mM glycine, 1.6mM SDS, 20% (v/v) methanol). The gel was rinsed in transfer buffer and placed upon 6 pieces of 3MM paper. The membrane was placed on the gel and topped with the remaining pieces of 3MM. Air bubbles were removed and transfer effected at full power for 15-40 minutes. The membrane was removed after the transfer was complete, and could be stored at 4°C if desired. The membrane was blocked for 30 minutes in blotto (2.5% non-fat milk powder, 0.1% NP40 in PBS) before incubation with antiserum in blotto (1:5000 dilution) for a minimum of 4 hours. The membrane was subsequently washed three times in blotto before incubation with anti-rabbit IgG conjugated to alkaline phosphatase (1:5000 in blotto) for 2 hours at room temperature. The membrane was washed extensively in blotto and alkaline phosphatase buffer (100mM Tris pH 9.5, 100mM NaCl,

5mM $MgCl_2$) before development with chromogenic alkaline phosphatase substrate. This substrate was prepared by dissolving 16mg nitroblue tetrazolium and 8mg of 5-bromo-4-chloro-3-indolyl phosphate in 50ml of alkaline phosphate buffer. Substrate solution was added to the membrane and allowed to developed as long as necessary before termination by rinsing in water.

Chapter 5: Materials

5.1 Tissue culture media and supplies

Supplier: Beatson Institute central services

Penicillin (7.5mg/ml)

Streptomycin (10mg/ml)

Sterile PBS

Sterile glassware and pipettes

Supplier: Fison Scientific equipment, Loughborough, Lies., England.

DMSO

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

Special liquid medium

Foetal calf serum

200mM glutamine

Sodium bicarbonate

Supplier: Northumbria Biologicals Ltd., Cramlington, England.

RPMI-1640 medium

Supplier: A/S Nunc, Roskilde, Denmark.

Tissue culture flasks

Nunc tubes

5.2 Bacterial media

Components of media were purchased from BDH Chemicals Ltd., Poole, Dorset, England, unless otherwise stated.

Supplier: Beatson Institute central services

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

Sterile glassware

Supplier: Difco, Detroit, Michigan, USA

Agar

Bacto-tryptone

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

Ampicillin

X-gal

5.3 Plasticware

Supplier: Becton Dickinson Labware, Plymouth, England.

Falcon tubes

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

All bacteriological dishes

30ml universal tubes

5.4 Kits, columns and miscellaneous

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: Nichols Institute Diagnostics Ltd., Saffron Walden, Essex, England.

Human growth hormone assay kit.

Supplier: NEN Research Products DuPont, Stevenage, Hertfordshire, England.

DNA sequencing system (Maxam & Gilbert).

Supplier: Perkin Elmer Cetus, Norwalk, CT

GeneAmp PCR reagent kit.

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

Nick columns

5.5 Membranes, paper and X-ray film.

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

Hybond N^+ nylon membrane

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

Dialysis tubing

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)

5.6 Nucleotides, polynucleotides, RNA and DNA.

The following nucleotides were used to label DNA and were purchased from Amersham International plc, Amersham, Bucks., England:

[α-^{32}P] dCTP, dATP, dTTP, dGTP 3000 Ci/mmol.

[α-^{35}S] dATP 1000 Ci/mmol.

[γ-^{32}P] dATP 5000 Ci/mmol.

Unlabelled nucleotides were also supplied by Amersham.

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

Poly [dI-dC]

Supplier: Sigma chemical company Co. Ltd., Poole, Dorset, England

Salmon sperm DNA

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

DNA markers bacteriophage θX174 DNA (HaeIII-cut) and bacteriophage l DNA (Hind III-cut).

RNA ladder 0.24-9.5kb.

5.7 Enzymes

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

All enzymes not included in the following list.

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

Calf intestinal alkaline phosphatase (1U/µl)

RNAseA

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

T4 polynucleotide kinase (10U/µl)

Klenow DNA polymerase (1U/µl)

Supplier: Promega, Madison, Wisconsin, USA.

RNasin (20U/µl)

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

Lysozyme

Supplier: Lorne Laboratories Ltd., Twyford, Reading, England.

DNAse1 (Molecular biology grade, RNase and protease free)

5.8 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

Diethylpyrocarbonate (DEPC)

Dithiothreitol

Nitroblue tetrazolium

MOPS

TPA

Retinoic acid

Spermidine

TEMED

Triton X-100

Xylene Cyanol

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, Northuberland, England.

Bovine Serum Albumin (20% w/v).

Supplier: Fisons Scientific Equitment, Loughborough, England.

Formaldehyde (38% w/v).

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

Formamide

5.9 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 6: Analysis of cell lines for the expression of the defensin genes

The defensin genes were previously found to be expressed in the HL60 cell line in this laboratory, but after initial experiments this expression was lost. A series of other haematopoietic cells were also screened to test for defensin expression, see table 6.1 (taken from R.Lamb PhD thesis). Although a large number of different myeloid cell lines were tested no expression could be detected, this included six different HL60 sublines.

Table 6.1: Cell lines screened for defensin gene expression

HL60p25	KG1
HL60 B	KG1A
HL60 T	Daudi
HL60 ICRF	Raji
HL60 N	CEM
K562	MOLT-4
ML1	Km3
U937	

This result is perhaps not surprising given the defensin genes very narrow window of expression during differentiation, as only the HL60 cell lines are at a stage of granulocytic differentiation which would be expected to express the defensin genes (Wiedemann *et al.*, 1989; Daher *et al.*, 1989; Koeffler 1991). One possible approach was to try to induce expression of the defensin genes with a range of differentiation inducing agents. Cell lines which are arrested at an earlier stage of differentiation than promeylocyte/myelocyte may switch on the defensin genes as they differentiate through this intermediate stage. To test this possibility, human

myeloid cell lines were induced to differentiate and RNA was harvested at different time points and northern blot analysis was undertaken to probe for defensin mRNA.

6.1 Analysis of defensin expression during HL60 differentiation

The first experiments were to determine if the defensin genes were expressed in the HL60 cell line after treatment with differentiation inducers. Three different inductions of HL60 were undertaken, with the differentiation inducers TPA (induces macrophage differentiation), DMSO and retinoic acid (induce granulocytic differentiation). RNA was harvested from a number of time points (see figures 6.1 and 6.2) and analysed by northern blot. In these experiments it is important to determine that the induction of differentiation was successful. To determine this, the differentiation status of the cells were assayed for the ability to reduce NBT, which is a marker of mature phagocytes and by morphological examination, as differentiation results in profound morphological change. For the TPA induction, 90% of the cells became adherent by 24hrs. The uninduced cultures contained 2% NBT-positive cells, fully induced retinoic, DMSO and TPA treated cultures contained 72%, 85% and 40% respectively. The low proportion of NBT-positive cells in the TPA treated cells is a reflection of a poorer ability of these cells to reduce NBT in comparison to their normal counterparts, rather than a failure of this particular induction (Koeffler *et al.*, 1981). These results indicated that the differentiation inductions were successful.

The results of the northern blot analysis using defensin cDNA as a probe can be seen for the DMSO, TPA and retinoic acid inductions in figures 6.1 and 6.2. For each of the inductions, no defensin mRNA could be detected, with the exception of the positive control which is RNA isolated from the peripheral leukocytes of a CML patient. To check for integrity and quantity of the RNA, the blots were reprobed with β_2-microglobulin (panel I in each figure) which remains constant during myeloid differentiation. Clearly the defensin genes are not expressed at appreciable levels

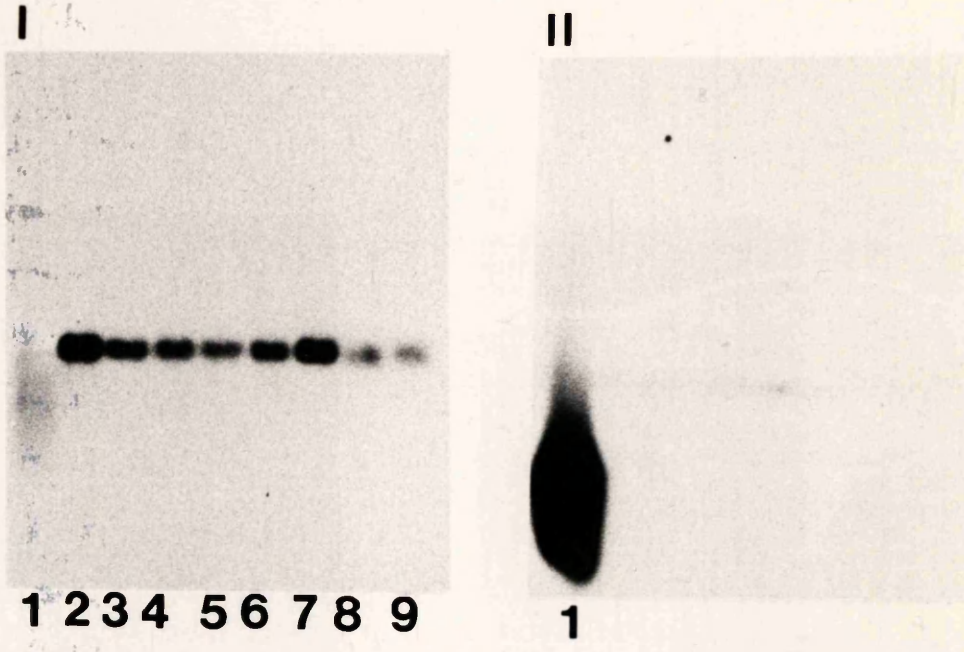
Figure 6.1A: Northern blot analysis of RNA extracted from an HL60 DMSO induced differentiation time course. Sample: 1, 5μg of CML RNA, all other lanes contain 15μg of RNA extracted from HL60 harvested after treatment with DMSO (final conc.1.5%) for :- **2**, 0 mins; **3**, 30 min; **4**, 1hr; **5**, 2hrs; **6**, 8hrs; **7**, 24hrs; **8**, 72hrs; **9**, 120hrs.

The filter was probed with both a defensin cDNA (panel II) and a β_2-microglobulin cDNA (panel I). The bands generated are of estimated size of 1.1 and 0.75 kd in panels I and II respectively. The defensin probe was a full length HNP3 encoding cDNA (Lamb 1989), and the β_2-microglobulin probe was a 545bp Pst1 fragment (Sugg *et al.*, 1981).

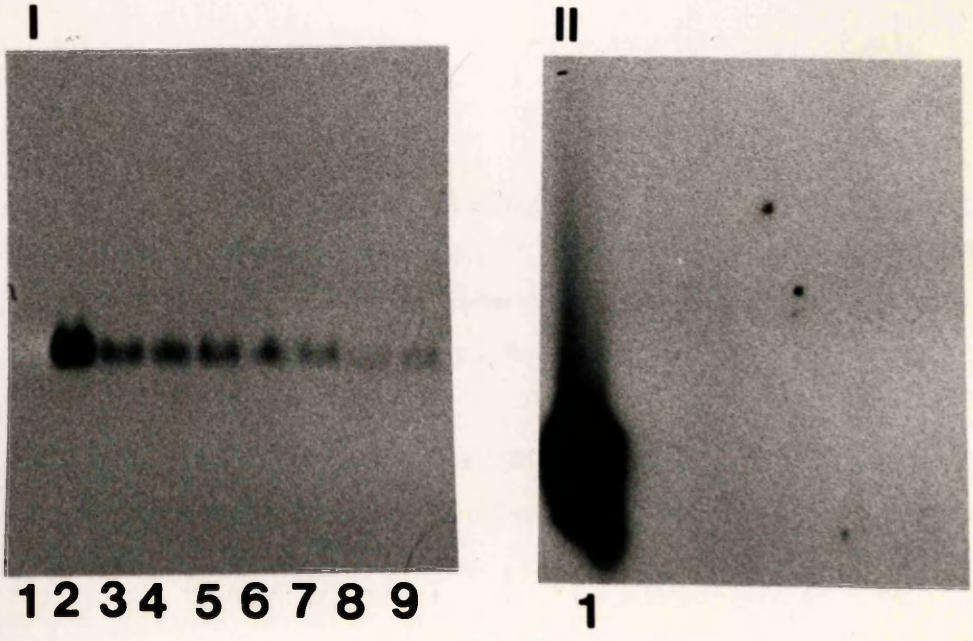
Figure 6.1B: Northern blot analysis of RNA extracted from an HL60 TPA induced differentiation time course. Sample: 1, 15μg of CML RNA, all other lanes contain 10μg of RNA extracted from HL60 harvested after treatment with TPA (final conc.10^{-7}M) for :- **2**, 0 mins; **3**, 30 min; **4**, 1hr; **5**, 2hrs; **6**, 8hrs; **7**, 24hrs; **8**, 72hrs.

The filter was probed with both a defensin cDNA (panel II) and a β_2-microglobulin cDNA (panel I). The bands generated are of estimated size of 1.1 and 0.75 kd in panels I and II respectively.

A



B



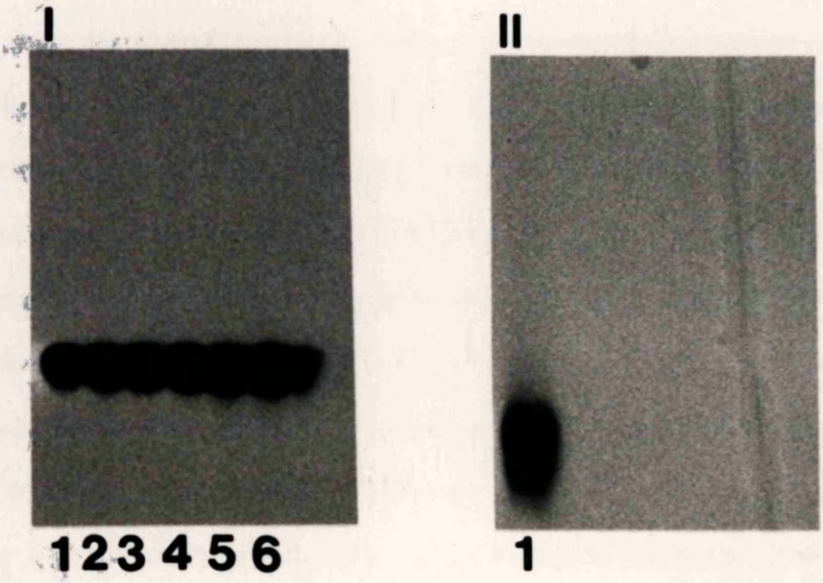


Figure 6.2: Northern blot analysis of RNA harvested during an HL60 retinoic acid induced differentiation time course. Sample: **1**, 15µg of CML RNA, all other lanes contain 15µg of RNA extracted from HL60 harvested after treatment with retinoic acid for:- **2**, 0 mins; **3**, 2hrs; **4**, 8hrs; **5**, 3 days; **6**, 5 days.

The filter was probed with both a defensin cDNA (panel II) and a β_2-microglobulin cDNA (panel I). The bands generated are of estimated size of 1.1 and 0.75 kd in panels I and II respectively.

during induced differentiation of HL60 cells utilising these agents.

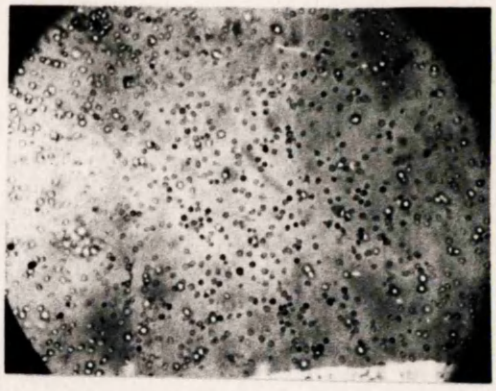
6.2 Analysis of defensin expression during ML1 induced differentiation.

This cell line is arrested at the myelomonoblast stage of differentiation, and can be induced to differentiate (Takeda et al., 1982; Craig et al., 1984). This differentiation can generate cells with characteristics of granulocytes or macrophages depending on the agent used (Takeda *et al.*, 1982). This cell line was induced with DMSO (which generates cells with granulocytic characteristics) and TPA (which generates monocytic like cells) and RNA was harvested at different time points. Differentiation was assessed by morphological examination and by NBT staining. This differentiation generated 25% and 55% NBT-positive cells after 7 days DMSO and 5 days TPA respectively which is similar to previously reported inductions of this cell line (Takeda *et al.*, 1982). TPA treatment resulted in rapid morphological changes, with greater than 75% of the cells becoming adherent by 12hrs (figure 6.3 A shows the morphological changes observed after 5 days culture with TPA).

To determine if the defensin genes were expressed during induced ML1 differentiation, northern blot analysis was undertaken using defensin cDNA as a probe. The results can be seen for the DMSO and TPA inductions in figures 6.3 B and 6.3 C respectively. No expression of the defensin genes can be detected during either TPA or DMSO induced ML1 differentiation (panel II in figure 6.3). To check for integrity and quantity of the RNA, the blots were reprobed with β_2-microglobulin (panel I in each figure).

A

Panel I



Panel II

Figure 6.3 A: Figure illustrating the morphological changes which occur during TPA induced ML1 differentiation. Panel I shows uninduced cells, panel II shows a culture of cells after 5 days incubation in TPA.

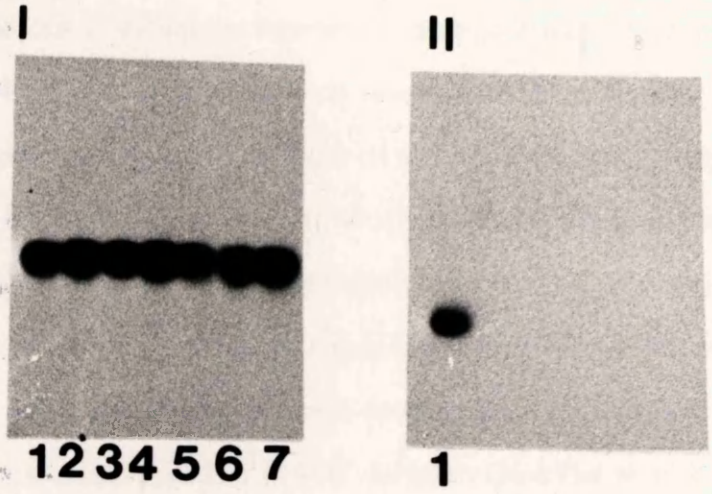
Figure 6.3 B: Northern blot analysis of RNA extracted from an ML1 TPA induced differentiation time course. Sample: 1; 15μg of CML RNA, all other lanes contain 15μg of RNA extracted from ML1 cells harvested after treatment with TPA ($5x10^{-10}$M) for:- **2**, 0 mins; **3**, 2hrs; **4**, 12hrs; **5**, 24hrs; **6**, 72hrs; **7**, 168hrs.

The filter was probed with both a defensin cDNA (panel II) and a β_2-microglobulin cDNA (panel I). The bands generated are of estimated size of 1.1 and 0.75 kd in panels I and II respectively.

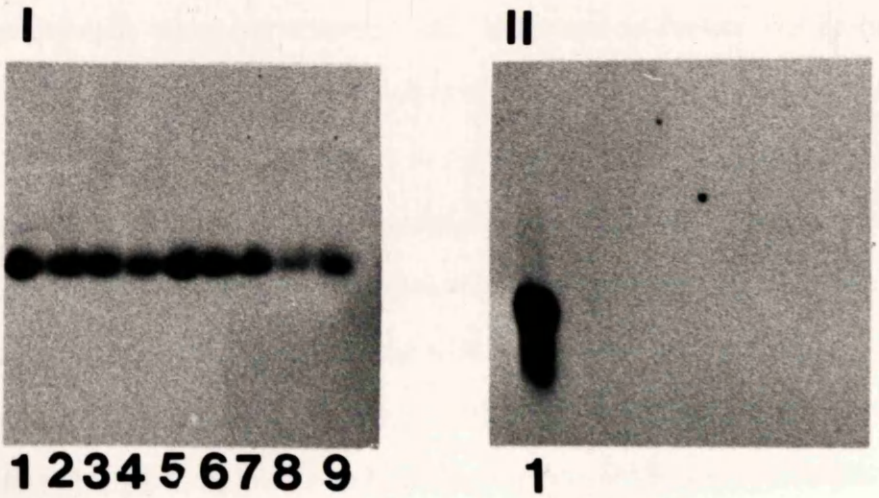
Figure 6.3 C: Northern blot analysis of RNA extracted from an ML1 DMSO induced differentiation time course. Sample: **1**, 15μg of CML RNA, all other lanes contain 15μg of RNA extracted from ML1 cells harvested after treatment with DMSO (final concentration 1.6%) for:- **2**, 0 mins; **3**, 2hrs; **4**, 8hrs; **5**, 12hrs; **6**, 24hrs; **7**, 72hrs; **8**, 120 hrs; **9**, 168hrs.

The filter was probed with both a defensin cDNA (panel II) and a β_2-microglobulin cDNA (panel I). The bands generated are of estimated size of 1.1 and 0.75 kd in panels I and II respectively.

B



C



6.3 Analysis of defensin expression during KG1 differentiation

This cell line is thought to represent a more primitive cell (myeloblast) than ML1 with 98% of the cells myeloblast-like and 2% intermediate to late stage granulocytes and rare macrophages. This cell line can proliferate in response to IL-3 and GM-CSF but these agents do not induce differentiation (Koeffler and Golde 1978). KG1 cultures however, can be induced to differentiate to macrophage like cells by the addition of TPA. This generates cells which become adherent, develop pseudopodia, phagocytic activity, non-specific acid and esterase activity, and expression of lysosomal enzymes and Fc receptors (Koeffler *et al.*, 1978b, 1981). Although the induction of differentiation results in macrophage-like cells, it is possible that these cells express some genes which are a component of the granulocytic program, given that the uninduced cultures are comprised of cells of the granulocytic lineage, and hence may express the defensin genes. To test this proposition a differentiation induction was undertaken and RNA was harvested at a range of time points. Differentiation was measured by morphological examination, with 80% of the cells becoming adherent after 12hrs and no further cell division occurring. In addition the cultures were assessed for the ability to reduce NBT and the fully induced culture was 37% positive in comparison to 3% positive before the induction. This figure is similar to previous reports indicating that the differentiation was successful (Koeffler *et al.*, 1981). Northern blot analysis was used to probe for the induction of defensin expression during KG1 differentiation. As can be seen in figure 6.4 no defensin mRNA can be detected during KG1 differentiation. The blot was reprobed with β_2-microglobulin to check the relative levels and the integrity of the RNA.

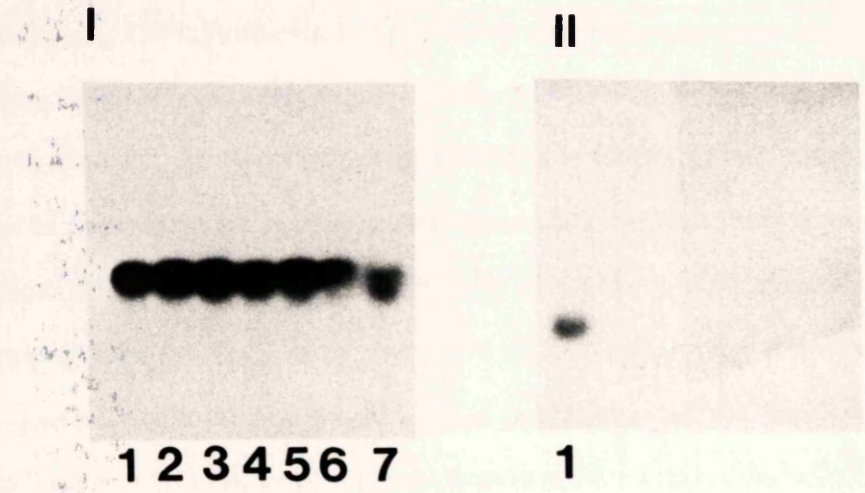


Figure 6.4: Northern blot analysis of RNA extracted from an KG1 TPA induced differentiation time course. Sample: **1**, 15µg of CML RNA, all other lanes contain 15µg of RNA extracted from KG1 cells harvested after treatment with TPA (10^{-7}M) for:- **2**, 0 mins; **3**, 2hrs; **4**, 8hrs; **5**, 24hrs; **6**, 48hrs; **7**, 72hrs.

The filter was probed with both a defensin cDNA (panel II) and a β_2-microglobulin cDNA (panel I). The bands generated are of estimated size of 1.1 and 0.75 kd in panels I and II respectively.

6.4 Analysis of the expression of the defensin genes during K562 differentiation.

This cell line was tested to see if differentiation could induce the expression of the defensin genes. Although this cell line has characteristics of immature erythroblasts, and are able to produce haemoglobin on induction of differentiation (Andersson *et al.*, 1979; Rutherford *et al.*, 1979), some granulopoietic markers are expressed on differentiation of these cells (Marie *et al.*, 1981). In addition, long term culturing in medium deprived of essential nutrients results in the generation of precursors of monocytic, granulocytic and erythrocytic cells (Lozzio *et al.*, 1981). This cell line most probably represents an early multipotent haemopoietic cell. As some granulopoietic markers are expressed on differentiation of this cell line, the defensin genes might be expressed, as a component of the granulopoietic differentiation program must be activated at least in a subfraction of the cells.

To test this possibility, a differentiation induction was carried out using sodium butyrate, and RNA was harvested at a range of time points (see to figure 6.5). The differentiation was not monitored by quantitative markers, however the induction of differentiation was clearly successful as in addition to observable changes in morphology the cell pellet becomes red in colour due to the production of haemoglobin by these cells. Again no expression of the defensin genes could be detected, although this is perhaps not surprising given the predominately erythroid phenotype of the differentiated cells.

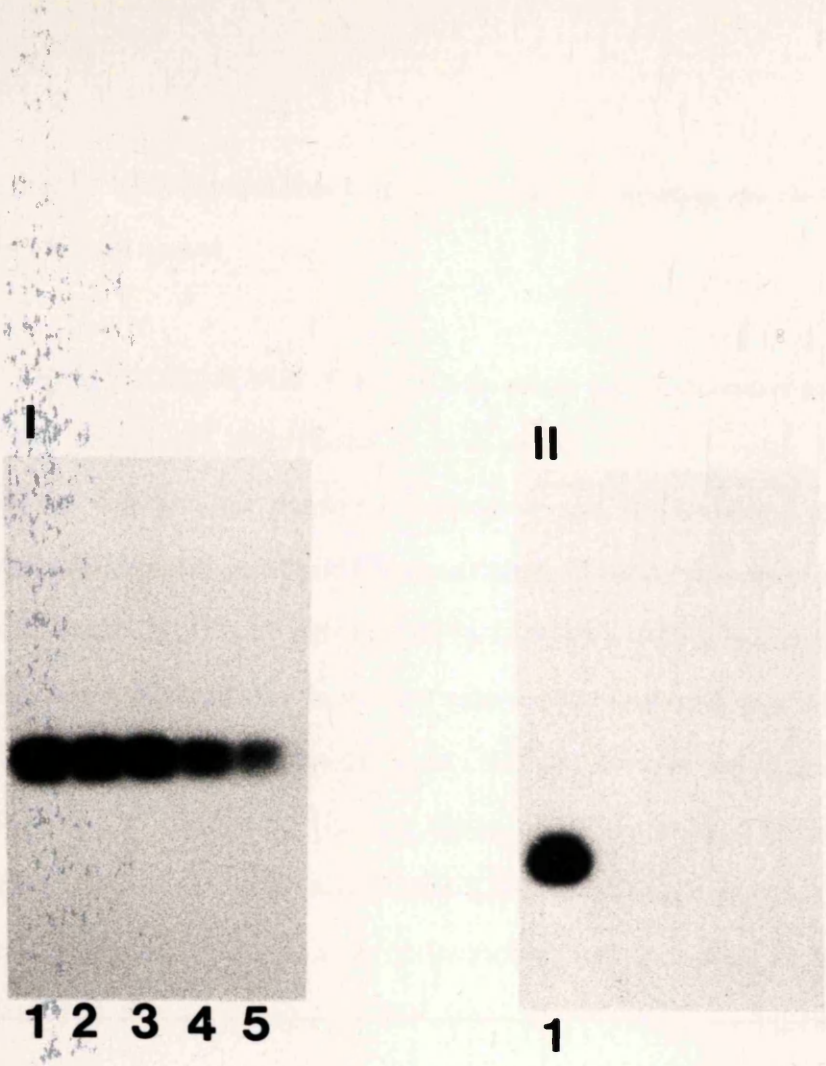


Figure 6.5: Northern blot analysis of RNA extracted from a K562 sodium butyrate induced differentiation time course. Sample: **1**, 15μg of CML RNA, all other lanes contain 15μg of RNA extracted from K562 cells harvested after treatment with sodium butyrate (10^{-7}M) for **2**, 0 mins; **3**, 2hrs; **4**, 24hrs; **5**, 72hrs

The filter was probed with both a defensin cDNA (panel II) and a β_2-microglobulin cDNA (panel I). The bands generated are of estimated size of 1.1 and 0.75 kd in panels I and II respectively.

6.6 Analysis of inflammatory agents ability to switch on the expression of the defensin genes

The loss of expression of the defensin genes was particularly puzzling given that it had previously been found to be expressed in these cells. One possible explanation was that the previous expression was the result of some kind of inflammatory response generated by some unknown factor present in the culture at that time. Evidence which lends credibility to this possibility is that in addition to defensins being expressed in immature neutrophils, defensin proteins have been identified in rabbit alveoli macrophages, and the level of these proteins can be increased by challenge with Freunds adjuvant (Lehrer *et al.*, 1981). To test this possibility HL60 cells were treated with LPS and adjuvant peptide (which is the immunologically active component of freunds adjuvant) to elicit an inflammatory response. RNA was harvested 2 hrs and 24 hrs post treatment with these agents (see figure 6.6 A) and expression of the defensin genes was assessed. No expression of the defensin genes could be detected, although no control to test if the LPS/adjuvant peptide had generated an inflammatory response was included in this experiment. However, the concentrations employed have been found to be effective by other investigators (Hanock *et al.*, 1987).

It could be that the uninduced HL60 cells are not capable of responding to the immunological insult as they represent an immature cell which is not fully functional. We would perhaps expect that if the expression of the defensin genes could be induced by an inflammatory reaction, this would be in a fully differentiated macrophage or neutrophil. A second experiment was undertaken to test if the differentiation state of the cells would effect its response to the adjuvant peptide and conversely if the peptide affected the differentiation state of the culture. HL60 cells were treated with either DMSO or TPA with or without adjuvant peptide. RNA and protein were harvested at a

Figure 6.6: The effect of inflammatory agents on defensin gene expression

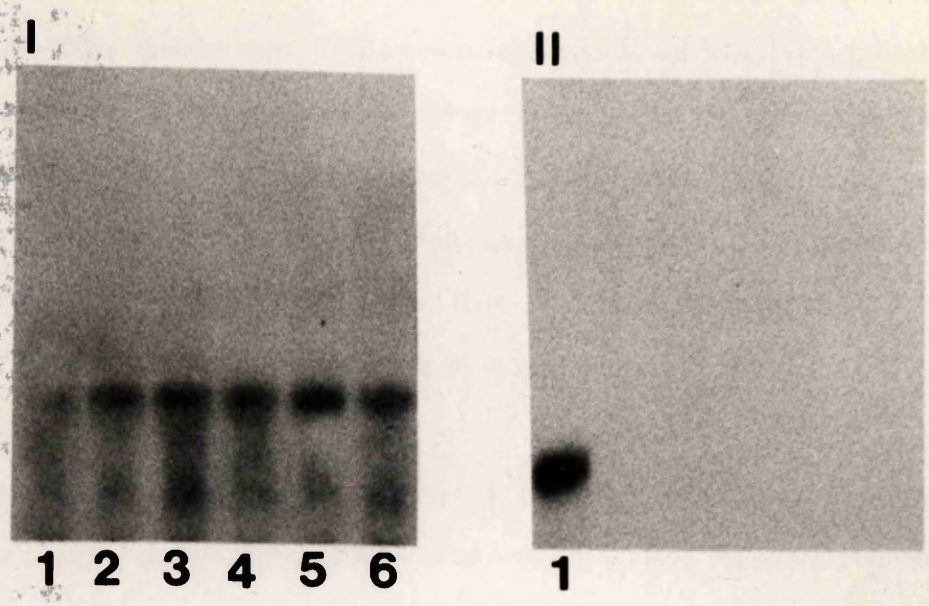
Figure 6.6 A: The effect of LPS and adjuvant peptide on defensin gene expression in HL60 cells. Sample: **1**, 15μg of CML RNA, all other lanes contain 15μg of RNA extracted from HL60 cells harvested after treatment with:- **2**, untreated; **3**, 2μg/ml LPS for 2hrs; **4**, 2μg/ml LPS for 24hrs; **5**, 2μg/ml adjuvant peptide for 2hrs; **6**, 2μg of adjuvant peptide for 24hrs.

The filter was probed with both a defensin cDNA (panel II) and a β_2-microglobulin cDNA (panel I). The bands generated are of estimated size of 1.1 and 0.75 kd in panels I and II respectively.

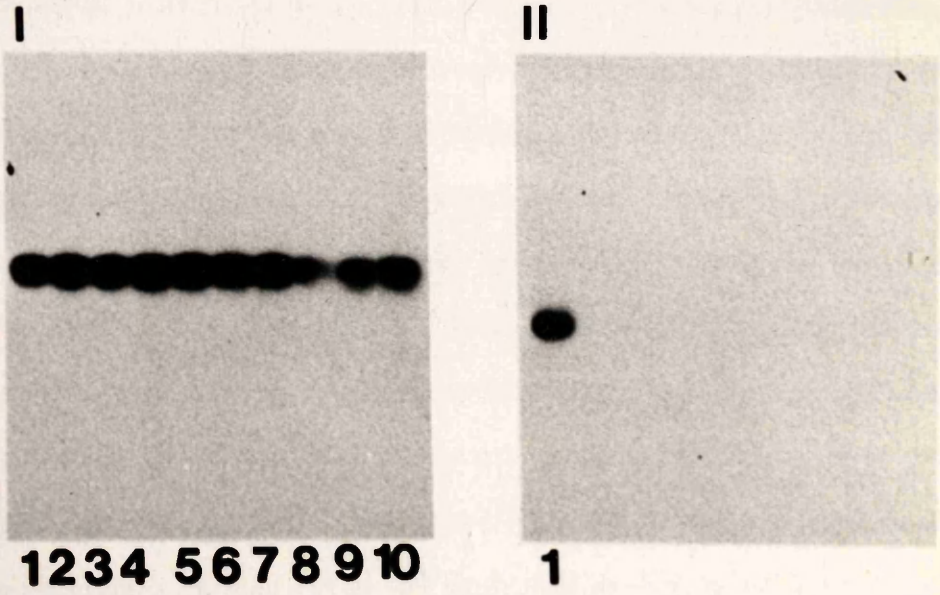
Figure 6.6 B: Northern blot analysis investigating the effect of adjuvant peptide on the expression of the defensin genes in HL60 cells induced to differentiate. Sample: **1**, 15μg of CML RNA, all other lanes contain 15ug of RNA extracted from HL60 cells harvested after treatment with:- **2**, untreated; **3**, DMSO and adjuvant peptide (2μg/ml) for 8hrs; **4**, DMSO and adjuvant peptide for 24hrs; **5**, DMSO and adjuvant peptide for 72hrs; **6**, DMSO and adjuvant peptide for 120hrs; **7**, TPA and adjuvant peptide for 8hrs; **8**, TPA for 24hrs; **9**, TPA and adjuvant peptide for 72hrs.

The filter was probed with both a defensin cDNA (panel II) and a β_2-microglobulin cDNA (panel I). The bands generated are of estimated size of 1.1 and 0.75 kd in panels I and II respectively.

A



B



range of time points and defensin message and protein was assessed by northern and western blotting. The differentiation state of the cultures was measured by NBT staining and morphological examination. The addition of the adjuvant peptide had no effect on the differentiation state of HL60 cells, although in the 3-day TPA-induced cells the presence of the agent seemed to activate the macrophage-like cells to engulf dead cells/debris in the culture (data not shown). As can be seen in figure 6.6 (panel II) no expression of the defensin genes could be detected. A positive control was included of CML RNA to indicate that the hybridisation was successful, and the filter was reprobed with β_2-microglobulin to determine the integrity of the RNA (panel I).

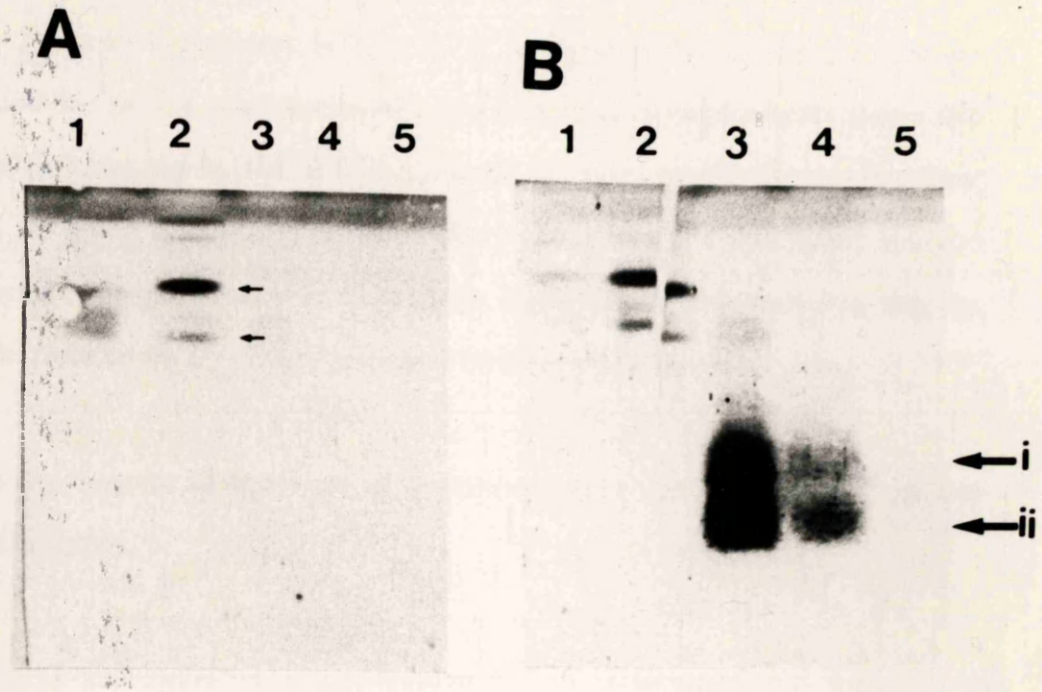
One possible explanation for the failure to detect defensin mRNA may be that in these cell lines it is particularly unstable. The defensin proteins themselves would be predicted to be stable as they are produced and stored prior to there use in the mature neutrophil. Hence a western blot may detect transient expression where a northern blot would not. A polyclonal antibody raised against a 10mer common to HNP1, HNP2 and HNP3 (Lamb, 1990) was used in a western blot to determine if defensin protein could be detected in HL60 cells. The ability of the defensin proteins to be detected by this immune serum is indicated by the positive control of normal peripheral blood leukocytes. This detects two low molecular weight bands (panel B, indicated by the arrows labelled i and ii), the lower band is of estimated molecular weight of 3kd, and is hence likely to correspond to the fully processed defensin proteins. The larger band is likely to be the unprocessed defensin which has a predicted molecular weight of 9kd, and the estimated molecular weight of the band on the gel is approximately 7-8kd, and could correspond to this precursor, and/or intermediately processed peptides (Valore and Ganz, 1992). These bands are specific as they are not detected by pre-immune serum (figure 6.7B panel A), however no defensin protein could be detected in HL60 cells (figure 6.7 B lane 2). The two bands indicated by the small arrows are non-specific as they are generated by both the pre-immune and immune serum. Having established the ability of the serum to be employed successfully to detect defensin

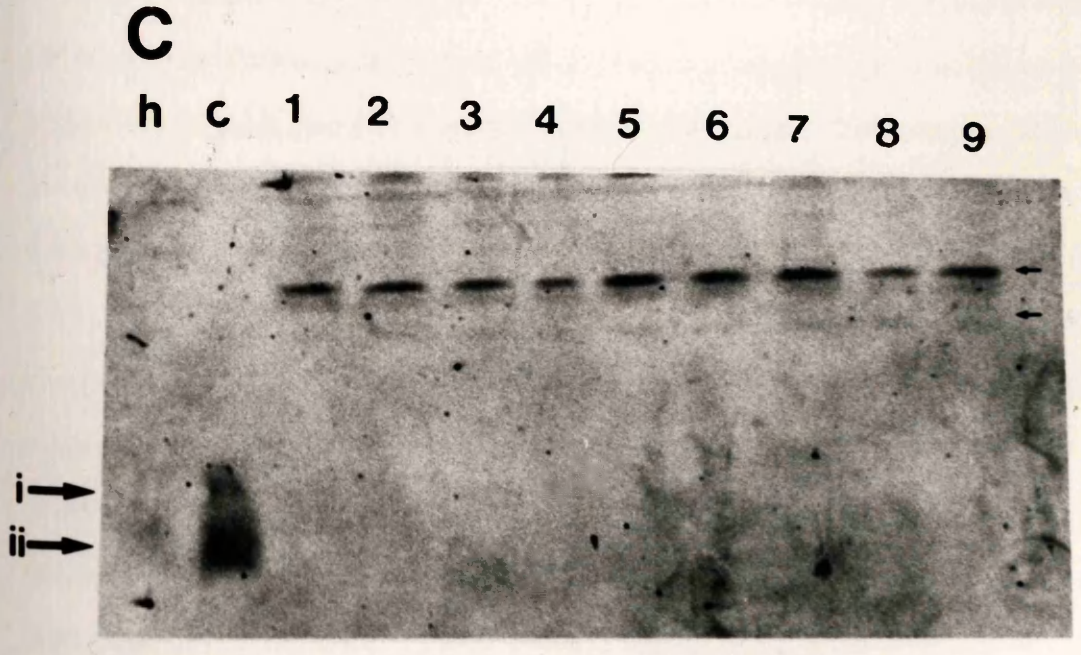
Figure 6.7 A and B: Western blot anlaysis to determine if HL60 cells contain defensin protein.

Immune serum raised against a 10mer common to HNP1, HNP2 and HNP3 was employed (Lamb, 1990) to probe western blots. Each sample contained total cellular protein from the following sources:- sample **1**, 20μg HeLa; 2, 20μg HL60; 3, 20μg periphal white blood cells (PWB); **4**, 2μg PWB; **5**, 0.2μg PWB.
Panel A and B represent duplicate filters, incubated with pre-immune and immune serum in A and B respectively. Bands which are specific to the immune serum are labelled i and ii.

Figure 6.7 C: Western blot analysis investigating the effect of adjuvant peptide on the expression of the defensin genes in HL60 cells induced to differentiate.

Immune serum raised against a 10mer common to HNP1, HNP2 and HNP3 was employed (Lamb, 1990) to probe western blots. Each sample contained total cellular protein from the following sources:- sample **h**, 20μg HeLa; **c**, 2μg PWB; **1**, 20μg uninduced HL60; **2**, HL60 cells incubated for 24 hrs with adjuvant peptide (2μg/ml); **3**, HL60 cells incubated for 72 hrs with adjuvant peptide (2μg/ml); **4**, HL60 cells incubated for 120 hrs with adjuvant peptide; **5**, HL60 cells incubated for 24 hrs with adjuvant peptide and DMSO; **6**, HL60 cells incubated for 72 hrs with adjuvant peptide and DMSO **7**, HL60 cells incubated for 120 hrs with adjuvant peptide and DMSO; **8**, HL60 cells incubated for 24 hrs with adjuvant peptide and DMSO; **9**, HL60 cells incubated for 72 hrs with adjuvant peptide and TPA.


A
1 2 3 4 5
B
1 2 3 4 5
i
ii


C
h c 1 2 3 4 5 6 7 8 9
i
ii

proteins, protein samples harvested simultaneosly to the mRNA (see figure 6.6) from the previously described experiment were analysed by western blot for the presence of defensin peptides.

The result is illustrated in figure 6.7 C, a negative control is protein from the HeLa cell line and the positive control is 2µg of normal white blood cell protein. No trace of defensin expression could be detected in any of the HL60 samples (20µg loaded per lane), despite allowing the alkaline phosphatase colourimetic assay to develop for several hours (hence the high background). This indicates that the defensin proteins are not present in these cells at detectable levels.

6.7 Investigation of the role of methylation in the 'switch off' of the defensin genes.

Any explanation as to why HL60 cells do not express defensin must take into account that there are two defensin genes (4 alleles), which would suggest that the mutation of defensin transcriptional control sequences is an unlikely method to switch off expression of these genes (Lamb, 1990). There is no apparent gross deletions in or around the defensin genes as tested by southern blot analysis. Given that mutation of the control regions of these genes is unlikely, what are the alternatives to account for the loss of expression? Methylation has been implicated in the repression of transcription, and could potentially generate epigenetic mutations by methylation of key transcriptional control regions. This could potentially occur at a much higher frequency than conventional mutation. It has been demonstrated that in tissue culture many genes become methylated, including many which remain constitutively unmethylated in all tissues *in vivo* (Antequera et al., 1990). This strongly implicates methylation in the repression of these genes in tissue culture. It has been estimated that 50% of CpG islands, which normally remain unmethylated *in vivo*, become methylated in tissue culture (Boyes and Bird, 1992). The methylation-mediated repression could be a possible mechanism to explain why

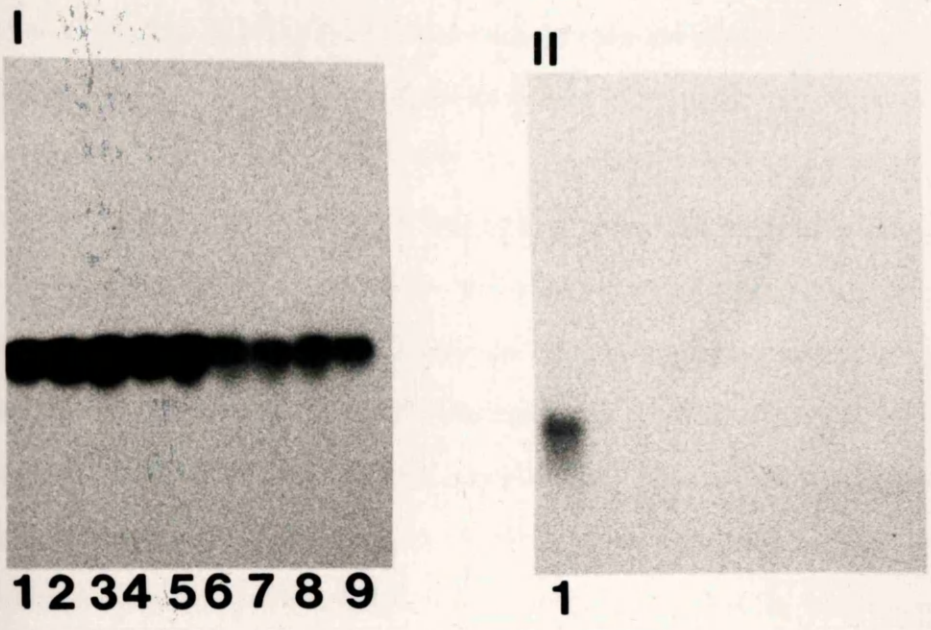


Figure 6.8: Northern blot analysis of RNA extracted from an HL60 cultures after treatment with 7μM 5-azacytidine. Sample: **1**, 15μg of CML RNA, all other lanes contain 15μg of RNA extracted from HL60 harvested after treatment with 5-azacytidine for:- **2**, 0 mins; **3**, 2hrs; **4**, 8hr; **5**, 24hrs; **6**, 48hrs; **7**, 72hrs; **8**, 120hrs; **9**, 144hrs.

The filter was probed with both a defensin cDNA (panel II) and a β_2-microglobulin cDNA (panel I). The bands generated are of estimated size of 1.1 and 0.75 kd in panels I and II respectively.

defensin gene expression is lost in cell lines. This could either be a direct effect by methylation of the defensin genes transcriptional control sequences or act indirectly via transcriptional repression of transcription factor(s) which are essential for expression of these genes.

To try to switch on the defensin genes, the demethylating agent 5-azacytidine was used to treat HL60 cells. This agent can induce differentiation of a number of myeloid (and non-myeloid) cell lines including HL60 cells. The HL60 cells were treated with 7μM 5-azacytidine as this was the highest concentration which was not cytotoxic (although some cell death was still apparent). The cells were cultured in the presence of 5-azacytidine for 7 days and RNA was harvested at a number of time points. The differentiation was examined by NBT staining (azacytidine induces granulocytic differentiation) and morphological examination. This revealed that only around 40% of the culture were NBT positive after 7 days in culture, suggesting that a large fraction of the cells were still undifferentiated. The levels of defensin mRNA were then assessed by northern blot analysis (figure 6.8 panel II), and no expression of the defensin genes could be detected. This result, however, does not rule out methylation as negatively regulating these genes in cell lines for a number of reasons. For example, the demethylation caused by this agent is not complete (i.e. the full genome is not demethylated); hence the defensin gene (or rather important transcriptional control regions) may not be demethylated. Another possibility is that the defensin genes are demethylated but this occurs after the cells are at a stage of differentiation at which expression of the defensin genes does not normally occur as a consequence of the differentiation inducing effect of 5 azacytidine. Hence, although the defensin genes are no longer repressed by methylation, positive acting transcription factors may no longer be present to activate the unmethylated defensin genes.

Classically the methylation status of genes has been analysed by comparing the southern blots of DNA digested with the HpaII and MspI. These restriction enzymes cleave DNA at CCGG, although MspI can cleave both CmCGG and CCGG, HpaII

Figure 6.9: Investigation of the methylation status of the DNA flanking the defensin genes

Figure 6.9 A: Southern blot analysis of the methylation status of the DNA flanking the defensin genes by HpaII and MspI digestion. Each lane contains 10μg of genomic DNA digested to completion with HpaII or MspI. The blot was probed with the 200bp fragment illustrated in figure 6.9 B. Sample **1**, normal peripheral lymphocytes; **2**; CML1 **3**, CML2; **4**, CML3; **5**, HL60. The lower band indicated by an arrow is of estimated molecular weight of 1.3kb. The upper band is of high molecular weight as there is little movement out of the well.

Differentiatial white cell counts of the CML samples:

CML1	CML2	CML3	
0	36	20	PMN
1	24	23	non-segmented bands
1	12	21	metamyelocytes
1	6	12	myelocytes
2	2	6	promyelocytes
90	1	2	myeloblasts
0	4	1	eosinophils
1	4	1	basophils
2	5	3	lymphocytes
0	4	5	monocytes
2	2	4	nucleated RBC

Figure 6.9 B: Control experiment to test if the digestions have gone to completion. From each of the digests described in figure legend 6.10 A, 1/10 of the reaction was taken and 4μg of of bluescript plasmid was added. The reaction was incubated for 12 hrs and the digests were analysed by southern blot using bluescript as a probe.

A
HpaII
MspI
lymph
HpaII
MspI
sample1
HpaII
MspI
sample2
HpaII
MspI
sample3
HpaII
MspI
HL60

B
HpaII
MspI
lymph
HpaII
MspI
sample1
HpaII
MspI
sample2
HpaII
MspI
sample3
HpaII
MspI
HL60

can only cleave the unmethylated DNA. Comparison of the restriction patterns generated after probing the region of interest gives an indication of whether this region of DNA is methylated. To test the hypothesis that the defensin genes are repressed by methylation as a result of propagation in tissue culture, comparison of the methylation status of the genes (and upstream regions) in a range of samples of genomic DNA were assessed by southern blot analysis after digestion with HpaII and MspI. The methylation pattern in CML samples known to express the defensin genes, HL60 cells and a sample of lymphocytes were investigated, to test if expression of the defensin genes could be correlated to a specific methylation pattern.

It is essential for this experiment that the digestion goes to completion as a direct comparison is being made between the two enzymes cleavage patterns. To determine that the digestions were complete, after the reactions were set up 1/10 was taken and 4µg of plasmid DNA was added. After incubation for 8 hrs these reactions were then ran on a gel, southern blotted and probed with plasmid DNA. The results can be seen in figure 6.9 B illustrating that all of the plasmid DNAs have been digested, indicating that the digests have gone to completion. The remaining 90% of the reaction was digested for a further 12 hrs after a second addition of enzyme (10U/µg of DNA). A small aliquot was ran out on a 1% agarose gel (figure 6.10 A), and the remaining was ran on a 2% agarose gel, southern blotted and hybridised (with the probe illustrated in figure 6.10 B) and the result can be seen in figure 6.9 A.

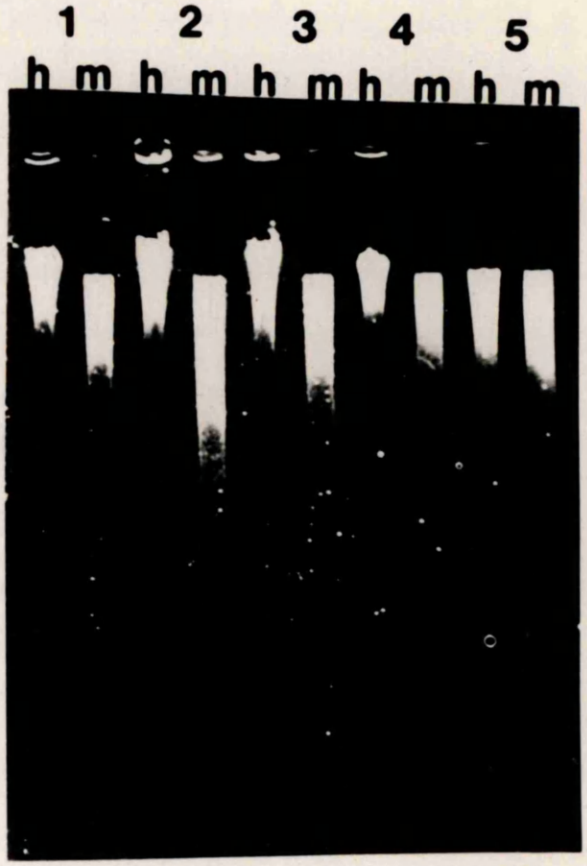
Consideration of the pattern generated by digestion of total genomic DNA from these samples, as seen in figure 6.10 A, illustrates that the HL60 genome is grossly undermethylated in comparison to that of the samples derived from the patients. This result is unlikely to represent a change in the amount of methylation seen during normal myeloid differentiation with the HL60 cell line simply representing a relatively immature cell. This is indicated as the leukaemic sample 1 contains 90% myeloblast cells but still shows a higher level of methylation than does the HL60 sample which is comprised of the more mature promyelocyte-like cells.

Figure 6.10: Investigation of the methylation status of the DNA flanking the defensin genes

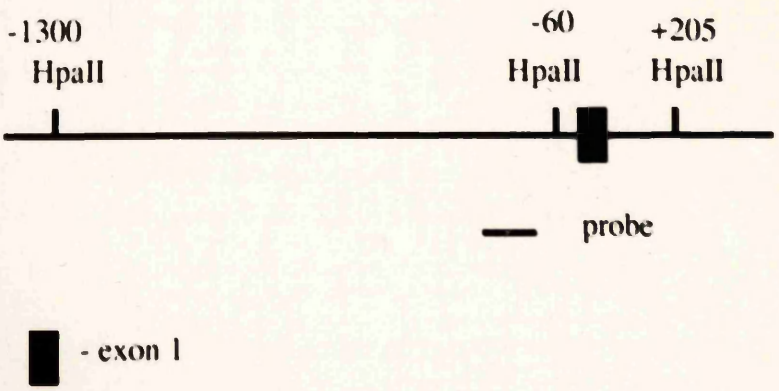
Figure 6.10 A: Ethidium stained gel of genomic DNA digested with HpaII or MspI. Samples of DNA were digested to completion with HpaII (lanes labelled h) and MspI (lanes labelled m), and 2μg was resolved on a 1% agarose gel. Sample **1**, normal peripheral lymphocytes **2**, CML1; **3**, CML2; **4**, CML3; **5**, HL60.

Figure 6.10 B: Diagram illustrating the probe used for the southern blot analysis as described in section 6.6 to investigate the methylation status of the defensin promoter. The numbers are relative to the main site of transcriptional initiation of the defensin genes.

A



B



Analysis of the southern blot of the gel after hybridisation to defensin promoter sequences demonstrated that this sequence in the leukaemic samples 2 and 3 were unmethylated, generating as expected a single band of 1.3kb (indicated by the arrow in figure 6.9 A) as the probe is within a 1.3kb HpaII fragment.. The DNA derived from lymphocytes and the HL60 cell line generated this 1.3kb band after digestion with Msp1, but failed to generate this band using HpaII which generated a higher molecular weight band/smear. In addition sample 1 also showed this digestion pattern, which is indicative of methylation (as a consequence of the inability of HpaII to cleave methylated sites) at the promoter regions.

This results suggests that the DNA derived from HL60, lymphocytes and a leukaemic sample containing 90% myeloblasts are methylated across the region analysed. However, the CML samples 2 and 3 which express the defensin genes at a high level are unmethylated or undermethylated over this region. As the higher molecular weight band has just migrated out of the well in the lympocyte and CML1 samples after HpaII digestion, we would predict that a number of HpaII sites must be methylated as this is a frequent cutter. This suggests that the defensin gene and/or surrounding sequences are methylated over a relatively large area. As the leukaemia sample 1 is comprised of immature blast cells, whereas samples 2 and 3 contain more mature cells, this suggests that the defensin genes are constitutively methylated and become demethylated only at a precise stage of differentiation in accord with their expression. Whether this methylation is causative in repression or simply a consequence of non-expression remains to be determined.

Chapter 7: DNAse1 hypersensitivity analysis

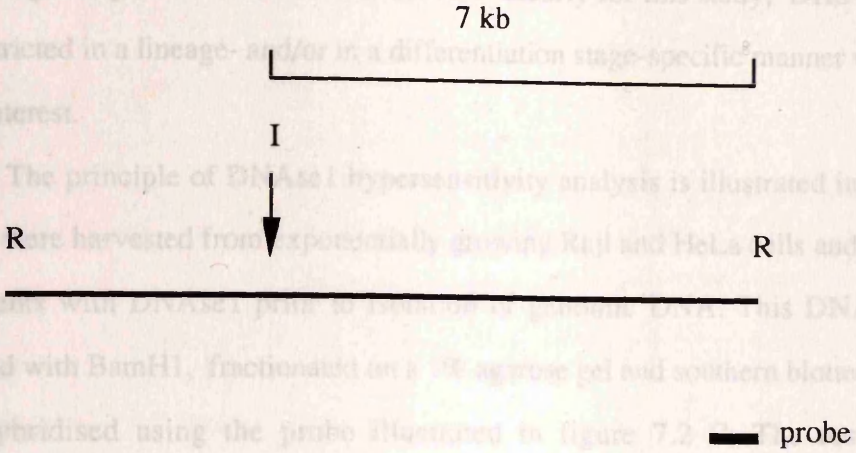
As no cell line tested could be induced to express the defensin genes an alternative approach was undertaken. The only cells available which expressed the defensin genes were peripheral leukocytes derived from CML patients. These samples contain varying proportions of myelocytes, in which defensin mRNA is particularly abundant and could thus be utilised as expressing cells in the following experiments (Weidemann *et al.*, 1989). One method of locating areas important in the transcriptional control of genes is to identify areas of chromatin which are hypersensitive to cleavage by DNAse1 (Wu *et al.*, 1979). This sensitivity to cleavage is a useful indicator, as sites of protein-DNA interaction are characterised by apparent gaps in the nucleosomal array. These sites often correspond to active promoter or enhancer elements (reviewed in Gross and Garrard 1988).

DNAse1 hypersensitivity analysis was employed to probe the chromatin structure surrounding the defensin genes to identify areas potentially important in the transcriptional regulation of these genes. A comparison of the chromatin structure encompassing the defensin genes from CML samples, myeloid and non-myeloid cell lines was undertaken to determine if any DNAse1 hypersensitive sites (DHS) could be identified. It would be of interest if any of these DHS were specific to the myeloid compartment and/or expressing cells, as these may be generated by transcription factors which are restricted in lineage- and/or differentiation stage-specific activity.

7.1 Analysis of the chromatin structure of the defensin genes in the HeLa and Raji cell lines.

The chromatin structure of the defensin genes in the epithelial (HeLa) and B-cell (Raji) cell lines was tested to determine if any DHS were present in cells outside the myeloid compartment. This is important as it is possible for a number of sites to be present in all tissues, under all conditions, irrespective of the expression of the

Figure 7.1: Principle of DNAse1 mapping



R - EcoR1 site

To identify DNAse1 hypersensitive sites, nuclei are isolated and briefly treated with nuclease (this brief treatment allows only 'hypersensitive' sites to be cleaved), the reaction is stopped and genomic DNA is isolated. To determine if any cleavage has occurred within a region of interest a southern blot is performed. Consider the following example (refer to figure). If the region to be analysed is flanked by EcoR1 sites, the DNA is digested to completion with this enzyme, the digested DNA is then run on a agarose gel and southern blotted. To map any hypersensitive sites a small probe whose 3' end is the EcoR1 site, is used in a hybridisation. If no DNAse1 cleavage had occurred within this region a single band of 10 kb would be expected. However, if a DNAse1 hypersensitive site was present at I, then a fraction of the DNA molecules would be cleaved at this site, then in addition to the 10kb band a 7kb band would also be generated. A smaller band of 3 kb is not detected as the probe is homologous only the 3' end of the EcoR1 fragment. This allows the DNAse1 hypersensitive site to be mapped with the size of the band generated equal to its distance from the 3' EcoR1 site.

gene. For example, the chicken lysozyme gene contains a number of constitutive DHS in addition to both inducible and tissue-specific sites (reviewed in Grewal *et al.*, 1992). These sites may be important in mediating the overall level of transcription, or perhaps organising a higher order chromatin domain. Clearly for this study, DHS sites which are restricted in a lineage- and/or in a differentiation stage-specific manner would be of most interest.

The principle of DNAse1 hypersensitivity analysis is illustrated in figure 7.1. Nuclei were harvested from exponentially growing Raji and HeLa cells and given brief treatments with DNAse1 prior to isolation of genomic DNA. This DNA was then digested with BamH1, fractionated on a 1% agarose gel and southern blotted. The filter was hybridised using the probe illustrated in figure 7.2 C. The results of the hybridisation can be seen in figure 7.2 A and B illustrating a single band in each lane of estimated size of 7.8kb, which would be predicted for a BamH1 digest when using this probe. No DHS can be detected over the region analysed. This region was 8kb, spanning the full length of the genes and approximately 5kb of upstream sequence (illustrated in figure 7.2 C), and was chosen for analysis as it was the full length of the genomic clone available. However, it should be remembered that transcriptional control elements can occur many kilobases up or downstream of the transcriptional start site and hence may not be identified by this analysis.

In addition to identifying DHS, it is possible to obtain an estimate of the relative sensitivity of the entire region to cleavage by DNAse1 in different cell types. This can be done qualitatively by comparing the reduction in intensity of the band (in this case the 8kb fragment) with increasing DNAse1. The lower the concentration of DNAse1 necessary to reduce the intensity of this band the greater the overall sensitivity of the region (providing that the amount of DNA loaded is even and the overall digestion is similar - i.e. comparison of the ethidium stains of the gels prior to blotting shows a similar level of cleavage). This analysis suggested that in these cell lines, this region of the chromatin was relatively insensitive to cleavage suggesting a condensed

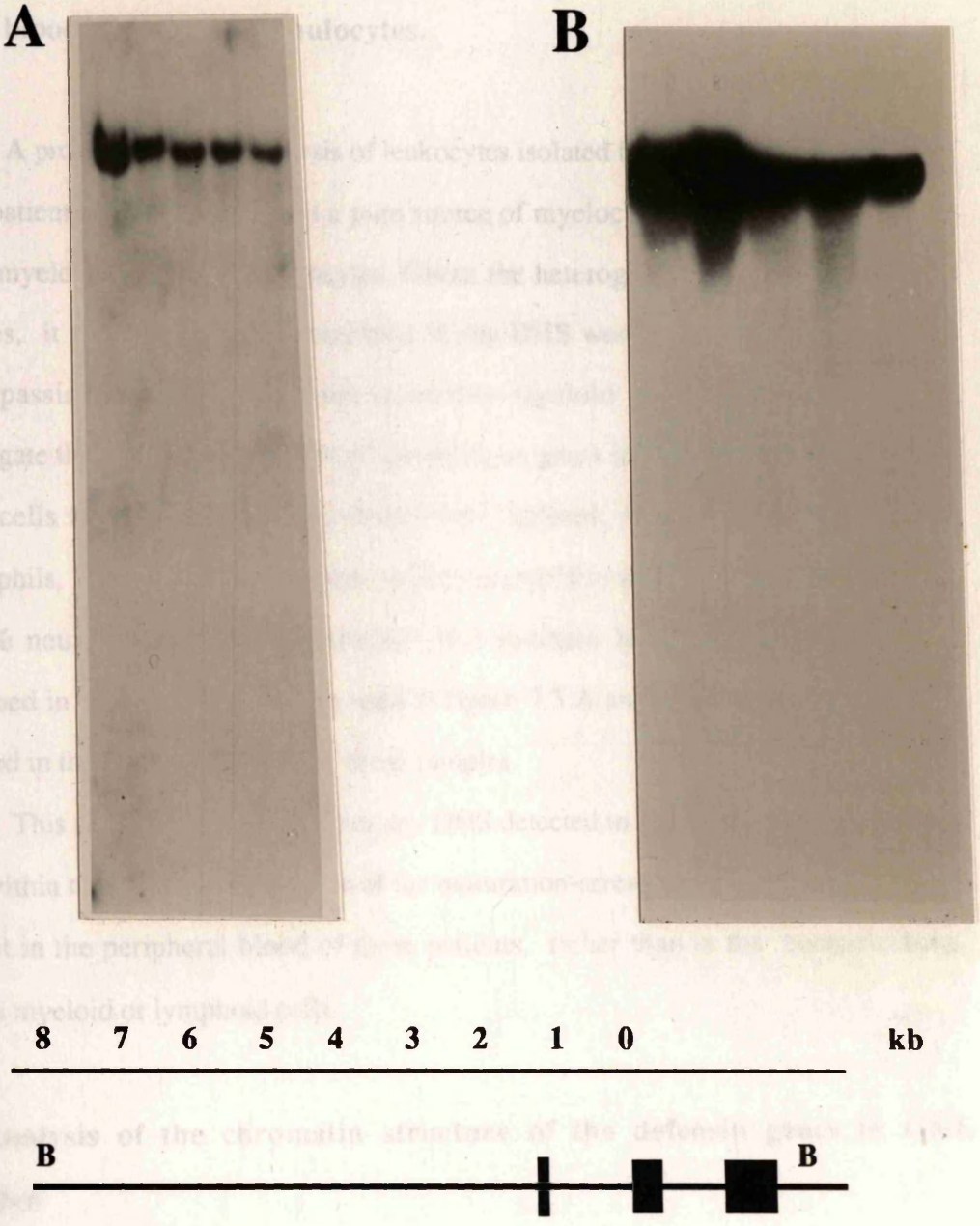
Figure 7.2 A:. Investigation of the chromatin structure spanning the defensin genes in the Raji cell line by DNAse1 hypersensitivity analysis.

After DNAse1 treatment of nuclei, DNA was harvested and 15μg was digested to completion with BamH1, run on a 1% agarose gel and southern blotted. The filter was hybridised to the probe illustrated in the cartoon in figure 7.2 C. Each lane corresponds to nuclei treated with DNAse1 at 37°C for 5 minutes at a concentration of: Lane **1**, mock DNAse1 treated; **2**, 1μg/ml; **3**, 2μg/ml; **4**, 4μg/ml; **5**, 8μg/ml.

Figure 7.2 B:. Investigation of the chromatin structure spanning the defensin genes in the HeLa cell line by DNAse1 hypersensitivity analysis.

After DNAse1 treatment of nuclei, DNA was harvested and 15μg was digested to completion with BamH1, run on a 1% agarose gel and southern blotted. The filter was hybridised to the probe illustrated in the cartoon in figure 7.2 C. Each lane corresponds to nuclei treated with DNAse1 at 37°C for 5 minutes at a concentration of: Lane **1**, mock DNAse1 treated; **2**, 1μg/ml; **3**, 2μg/ml; **4**, 4μg/ml; **5**, 8μg/ml.

Figure 7.2 C: Cartoon illustrating the region of chromatin analysed in this experiment. The solid boxes represent the defensin gene exons. The probe is 1 kb long and is flanked by the 3' BamH1 site (BamH1-HindIII fragment).


A
B
8 7 6 5 4 3 2 1 0 kb
B
B
B BamH1
Probe

chromatin organisation. This is discussed in more detail in section 7.3.

7.2 Analysis of the chromatin structure of the defensin genes in normal white blood cells and granulocytes.

A problem with the analysis of leukocytes isolated from the peripheral blood of CML patients is that they are not a pure source of myelocytes, and will contain later stage myeloid cells and lymphocytes. Given the heterogeneous nature of the CML samples, it was necessary to determine if any DHS were present in the chromatin encompassing the defensin genes in mature myeloid or in lymphoid cells. To investigate the chromatin structure of the defensin genes in normal blood cells, white blood cells from a normal blood donor were isolated. And as a source of mature neutrophils, a sample from a patient with a neutrophilia was analysed (this consisted of 92% neutrophils). DNAse1 analysis and southern blotting was carried out as described in figure 7.3. As can be seen in figure 7.3 A and B no DHS sites could be detected in this region for either of these samples.

This result would suggest that any DHS detected in the CML samples represent sites within the chromatin structure of the maturation-arrested immature myeloid cells present in the peripheral blood of these patients, rather than in the 'contaminating' mature myeloid or lymphoid cells.

7.3 Analysis of the chromatin structure of the defensin genes in CML samples

A small number of fresh samples of peripheral blood from patients with chronic myeloid leukaemia were available for analysis by DNAse1 hypersensitivity. Given that over the region of chromatin analysed, no DNAse1 hypersensitive sites could be detected in either normal leukocytes, epithelial (HeLa) or lymphoid (Raji) cells, it is likely that any DNAse1 hypersensitive sites identified are present in the

Figure 7.3: Investigation of the chromatin structure spanning the defensin genes in peripheral leukocytes by DNAse1 hypersensitivity analysis.

Figure 7.3 A: DNAse1 analysis of peripheral leukocytes from a neutrophilia patient (sample contained 92% neutrophils).

After the removal of erythrocytes from the sample and DNAse1 treatment of nuclei, DNA was harvested and 15µg was digested to completion with BamH1, electrophoresed on a 1% agarose gel and southern blotted. The filter was hybridised using the probe illustrated in the cartoon in figure 7.3 C. Each lane corresponds to nuclei treated with DNAse1 at 37°C for 5 minutes at a concentration of: Lane **1**, mock DNAse1 treated; **2**, 0.2 µg/ml DNAse1; **3**, 1µg/ml; **4**, 2µg/ml; **5**, 4µg/ml; **6**, 8µg/ml.

Figure 7.3 B: DNAse1 analysis of peripheral leukocytes from a normal blood donor.

After the removal of erythrocytes from the sample and DNAse1 treatment of nuclei, DNA was harvested and 15µg was digested to completion with BamH1, electrophoresed on a 1% agarose gel and southern blotted. The filter was hybridised using the probe illustrated in the cartoon in figure 7.3 C. Each lane corresponds to nuclei treated with DNAse1 at 37°C for 5 minutes at a concentration of: Lane **1** mock DNAse1 treated; **2**, 0.2 µg/ml DNAse1; **3**, 1µg/ml; **4**, 2µg/ml; **5**, 4µg/ml; **6**, 8µg/ml; **7**, 16µg/ml.

Figure 7.3 C: Cartoon illustrating the region of chromatin analysed in this experiment. The solid boxes represent the defensin gene exons.

A

Neutrophils

1 2 3 4 5 6

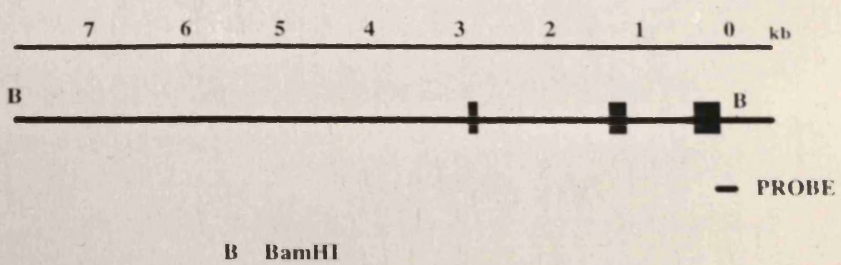
B

WBC

DNAse1

1 2 3 4 5 6

C



B BamHI

Figure 7.4 A:. Investigation of the chromatin structure spanning the defensin genes in the peripheral leukocytes from a CML patient (CML*A*).

After the removal of erythrocytes from the sample and DNAse1 treatment of nuclei, DNA was harvested and 15µg was digested to completion with BamH1, electrophoresed on a 1% agarose gel and southern blotted. The filter was hybridised using the probe illustrated in the cartoon in figure 7.4 B. Each lane corresponds to nuclei treated with DNAse1 at 37°C for 5 minutes at a concentration of: Lane **1**, mock DNAse1 treated; **2**, 1µg/ml; **3**, 2µg/ml; **4**, 4µg/ml; **5**, 8µg/ml.

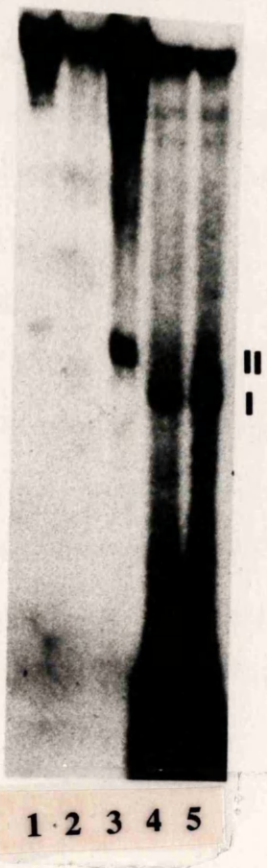
Differential white cell counts of the CML sample:

CMLA

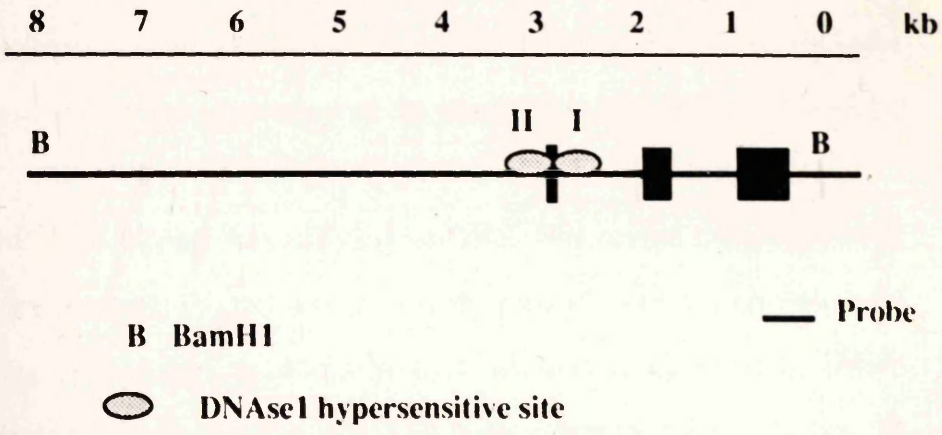
20	PMN
12	non-segmented bands
17	metamyelocytes
24	myelocytes
8	promyelocytes
4	myeloblasts
4	eosinophils
2	basophils
4	lymphocytes
3	monocytes
2	nucleated RBC

Figure 7.4 B: Cartoon illustrating the region of chromatin analysed in this experiment. The solid boxes represent the defensin gene exons and the open circles represent areas of enhanced cleavage by DNAse1.

A


II
I
1 2 3 4 5

B


8 7 6 5 4 3 2 1 0 kb
B
II I
B
Probe
B BamH1
DNAse1 hypersensitive site

immature myeloid cells present in these samples. The CML sample (CML*A*) which contained the highest proportion of myelocytes (41% myelocytes/metamyelocytes), was analysed for DHS as described in the previous experiments and the result can be seen in figure 7.4 A. This reveals the presence of two major sites labelled I and II. These sites map to span the immediate promoter and sequences within the first intron (as illustrated in figure 7.4 B). The site I is more sensitive to cleavage as this appears after DNAse1 treatment of nuclei at a concentration of 0.5μg/ml, whereas the site II appears only after treatment with 1μg/ml DNAse1 (and this concentration of DNAse1 seems to have generated a much greater amount of digestion than does 0.5μg/ml - see figure 7.6 C). Comparison between the ethidium stain gels of CML*A* and HeLa reveals that the chromatin of this region is more sensitive in the CML*A* samples (compare figures 7.6 C and A). As nuclei treated with 2μg/ml of DNA show a similar level of sensitivity to cleavage when ethidium stained gels are compared, however, when blotted and probed with a defensin genomic probe, the HeLa DNA is relatively uncleaved in comparison to the CML sample.

In addition to the two major bands labelled I and II there are two minor bands indicated just below the genomic 7.8 kb band. These bands are very faint and may represent cross hybridisation to a related sequence. An alternative explanation is that these are DHS present in cells which comprise a very minor component of the sample, perhaps more immature cells than myelocytes. However without purifying different populations of cells, further assessment of the significance of these sites cannot be undertaken.

A second CML sample was analysed for DHS. This sample is termed CML*B* (15% myelocytes/metamyelocytes) and has a lower proportion of myelocytes, with the sample being characterised by relatively more mature myeloid cells than CML*A*. The DHS analysis was carried out as described in the legend to figure 7.5, and the result can be seen in figure 7.5 A. The two arrows indicate the sites of enhanced cleavage, which map to the positions indicated in figure 7.5 B. These two sites are of equal intensity as both appear after treatment of nuclei with 0.5μg of

Figure 7.5 A:. Investigation of the chromatin structure spanning the defensin genes in the peripheral leukocytes from a CML patient (CMLB).

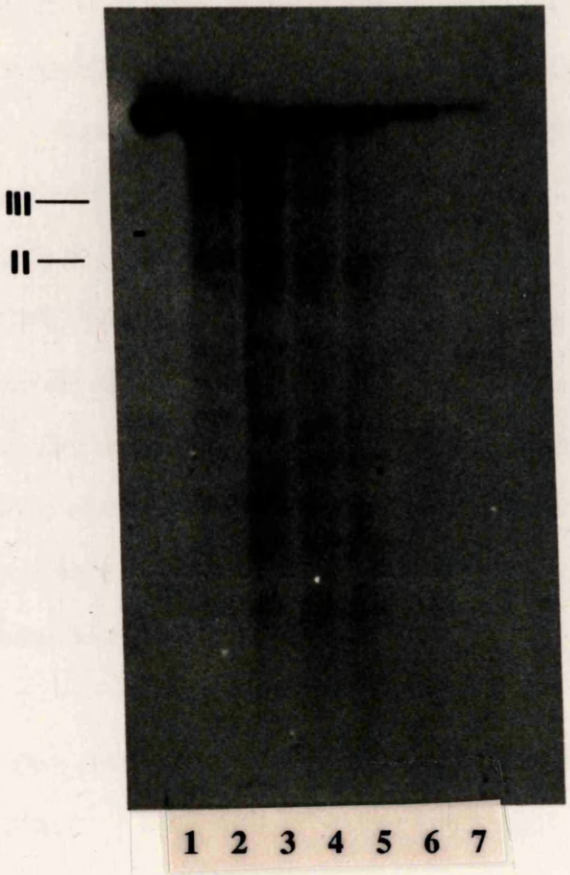
After the removal of erythrocytes from the sample and DNAse1 treatment of nuclei, DNA was harvested and 15µg was digested to completion with BamH1, electrophoresed on a 1% agarose gel and southern blotted. The filter was hybridised using the probe illustrated in the cartoon in figure 7.5 B. Each lane corresponds to nuclei treated with DNAse1 at 37°C for 5 minutes at a concentration of: Lane **1**, mock DNAse1 treated; **2**, 1µg/ml; **3**, 2µg/ml; **4**, 4µg/ml; **5**, 8µg/ml.

Differential white cell counts of the CML sample:

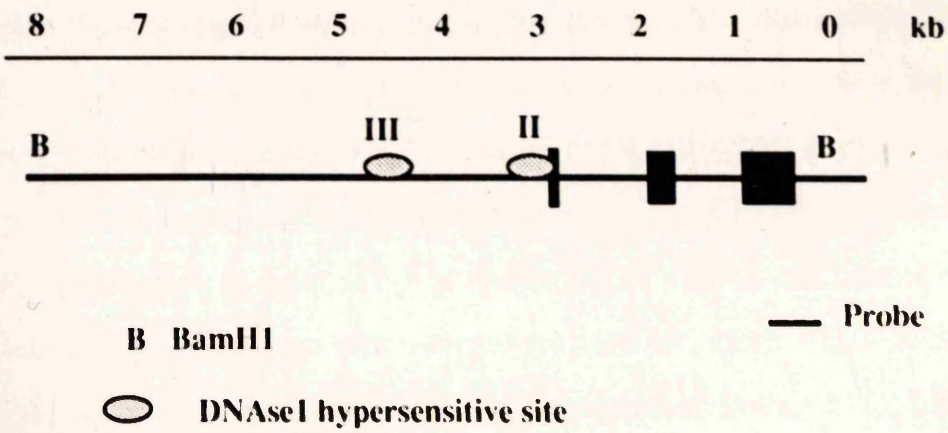
CMLB	
35	PMN
30	non-segmented bands
6	metamyelocytes
9	myelocytes
2	promyelocytes
0	myeloblasts
3	eosinophils
5	basophils
5	lymphocytes
2	monocytes
3	nucleated RBC

Figure 7.5 B: Cartoon illustrating the region of chromatin analysed in this experiment . The solid boxes represent the defensin gene exons and the open circles represent areas of enhanced cleavage by DNAse1.

A


III
II
1 2 3 4 5 6 7

B


8 7 6 5 4 3 2 1 0 kb
B
III
II
B
Probe
B BamHI
DNAse1 hypersensitive site

DNAse1 (lane 2). It seems that both of these sites are relatively weak in comparison to the DHS in CMLA, as there is a general background smearing on which these sites are superimposed. This may be a result of the mixed nature of the sample with the DHS present in only a sub-population of the cells (perhaps the myelocytes). Lending weight to this proposition is that although the DHS may be apparently weak, they are generated by a relatively low concentration of DNAse1 - suggesting they are hypersensitive but only in a fraction of the cells. Also of note is a series of faint bands below the marked DHS, present in lanes 3, 4 and 5 resembling a nucleosomal ladder. This suggests a depletion of H1 over this region - consistent with this area spanning a site of transcription. Depletion of H1 is often associated with active genes (Wolffe 1990; Garrard 1989), however the level of nuclease cleavage necessary to generate a nucleosomal ladder is higher than that necessary for DHS generation, and these sites are unlikely to represent areas of protein-DNA interaction.

7.4 Analysis of the chromatin structure of the defensin genes in HL60 and HL60gran cells.

As a number of DNAse1 hypersensitive sites had been identified in the CML samples, it was of interest to determine if the HL60 cell line contained any of these sites despite the lack of any defensin mRNA in these cells. This would address the question of whether these sites were present only in expressing cells, or if they occurred in myeloid cells irrespective of the expression of the genes. DHS analysis was carried out on uninduced and fully induced granulocytic cultures of HL60 (HL60gran) as described in the legend to figure 7.7. The results of this analysis can be seen in figure 7.7. Interestingly, the induced cells seem to have a similar pattern of DHS as the sample CMLA, however these sites are very weak in comparison. Absence of the DHS in the uninduced culture could be argued to be simply a result of loading differences between the samples. Longer exposure of the filter did reveal the presence of the site marked by the major arrow, although it was extremely weak in comparison.

Figure 7.6: Ethidium bromide stains of DNAse1 hypersensitivity gels comparing the HeLa cell line and CML and leukocyte samples.

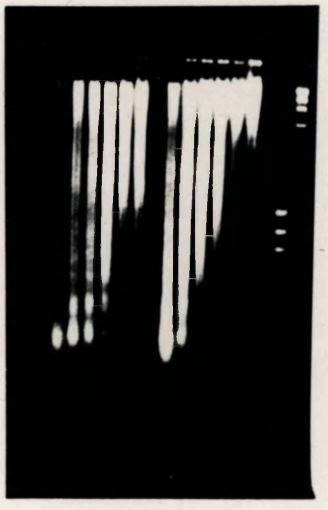
The figures correspond to the ethidium stained gels of the blots illustrated in figures 7.2 B, 7.3 A and B, 7.4 and 7.5.

A: HeLa cell line (figure 7.2 B)
B: normal leukocytes/granulocytes (figure 7.3 A and 7.3 B)
C: CML*A* (figure 7.4)
D: CMLB (figure 7.5)

Note each ethidium stained gel is inverted with respect to the southern blots, with the lane representing mock DNAse1 treated nuclei on the right of the page in this figure.

A HeLa

B WBC neutrophils



C CMLA

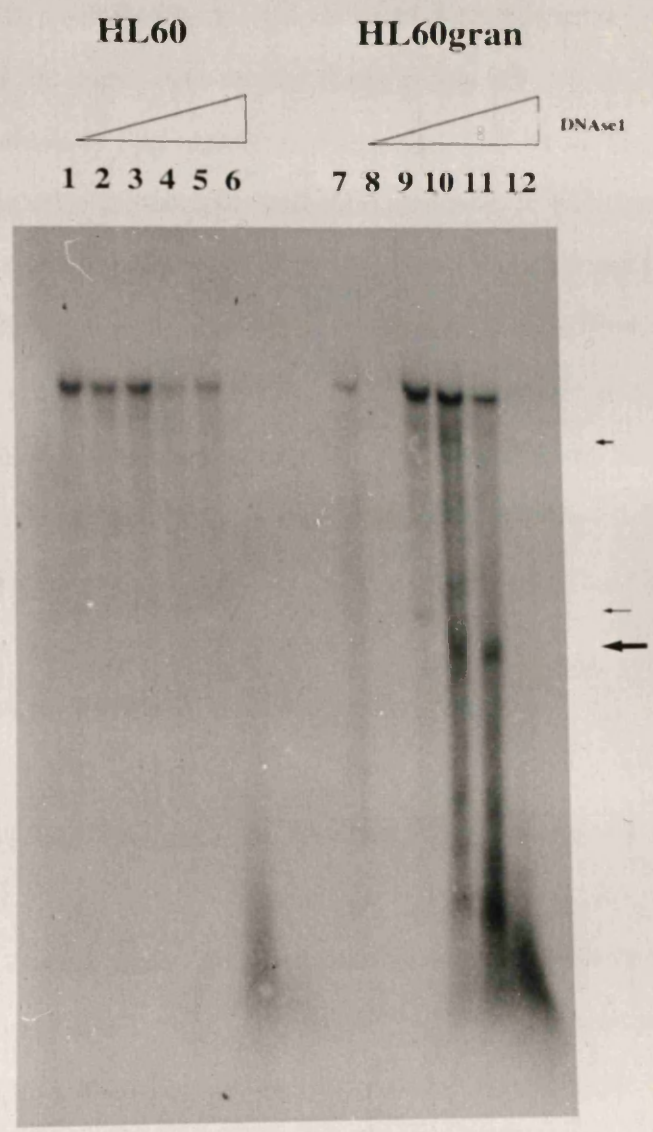
D CMLB

Figure 7.7 A:. Investigation of the chromatin structure spanning the defensin genes in the HL60 cell line before and after granulocytic differentiation.

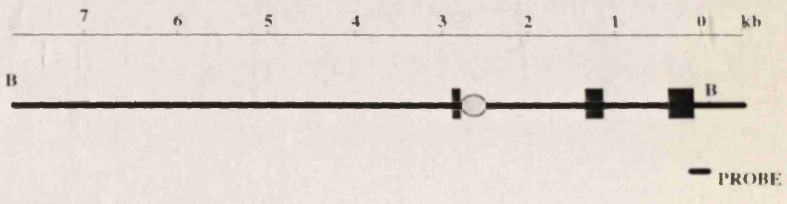
Differentiation of the HL60 culture with retinoic acid was performed as described in chapter 5. After DNAse1 treatment of nuclei, DNA was harvested and 15µg was digested to completion with BamH1, run on a 1% agarose gel and southern blotted. The filter was hybridised to the probe illustrated in the cartoon in figure 7.7 B. Each lane corresponds to nuclei treated with DNAse1 at 37°C for 5 minutes at a concentration of: Lane **1 and 7,** mock DNAse1 treated; **2 and 8,** 0.5ug/ml; **3 and 9,** 1µg/ml; **4 and 10,** 2µg/ml; **5 and 11,** 4µg/ml; **6 and 12,** 8µg/ml. The arrows indicate areas of enhanced susceptibility to DNAse1 cleavage.

Figure 7.7 B: Cartoon illustrating the region of chromatin analysed in this experiment. The solid boxes represent the defensin gene exons and the open circle represents an area of enhanced cleavage by DNAse1.

A



B



DNAse1 hypersensitive site

B BamHI

Another point of note is that in the induced culture, the mock DNAse1-treated sample shows some cleavage. This is likely to be the result of some endogenous nuclease activity which may be upregulated as a result of differentiation of these cultures. The significance of the hypersensitive sites found in this cell line are unclear given that this site is not particularly hypersensitive to cleavage. This would imply that only a sub-population of the cells contain this chromatin structure. In addition, why the induced cells have these sites is unknown, given that they are not present in cells from a neutrophilia patient. This could be a result of the defective differentiation program of HL60 cells, or that a fraction of the cells are undifferentiated or partially differentiated (i.e. only around 70% of cells become NBT-positive after the induction) and it is in these cells that contain the DHS. A similar experiment using HL60 cells after a shorter induction period, however, failed to detect any DHS (data not shown).

General conclusions from DNAse1 hypersensitive studies.

- No DHS could be identified outside of the myeloid compartment, or in fully differentiated myeloid cells.
- Three sites of potential interest were identified, the immediate promoter, a site within the first intron and an upstream region (see figure 7.8). These sites were present in the CML samples, although all three do not co-exist in the same sample, suggesting they do not all occur at the same stage of differentiation.

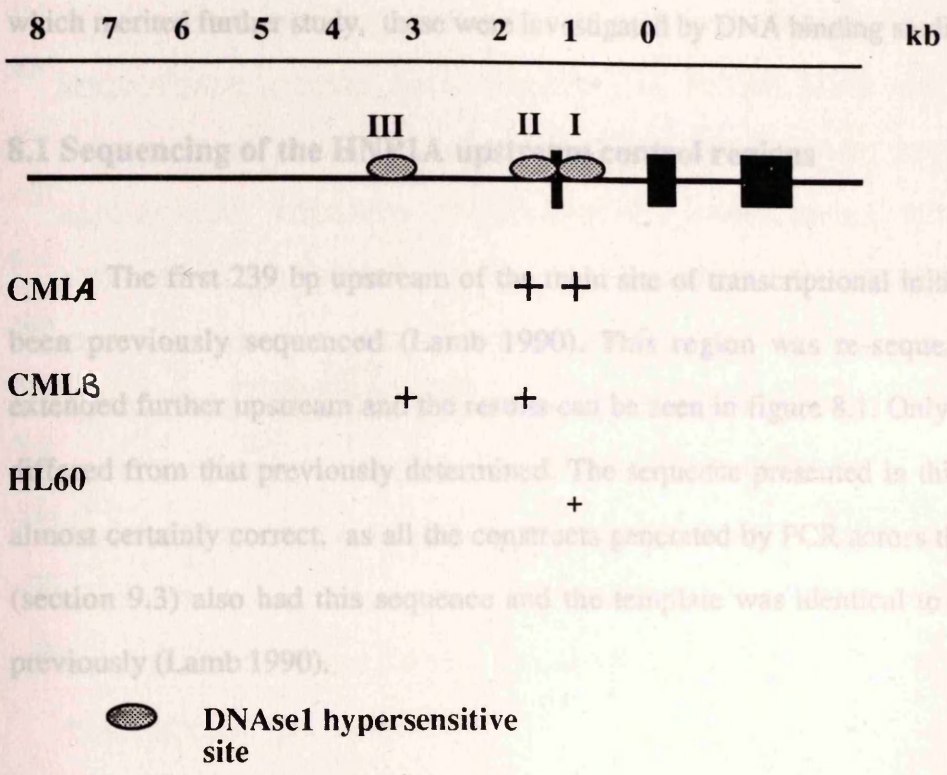


Figure 7.8: Summary of DNAse1 hypersensitive sites identified. Cartoon indicating the structure of the defensin genes and the sites to which the DHS were mapped. The plus sign indicates the presence of a DHS in the cell line/ sample indicated.

Chapter 8: *In vitro* binding studies

As the DNAse1 hypersensitivity analysis suggested two important sites which merited further study, these were investigated by DNA binding studies.

8.1 Sequencing of the HNP1A upstream control regions

The first 239 bp upstream of the main site of transcriptional initiation has been previously sequenced (Lamb 1990). This region was re-sequenced and extended further upstream and the results can be seen in figure 8.1. Only one base differed from that previously determined. The sequence presented in this work is almost certainly correct, as all the constructs generated by PCR across this region (section 9.3) also had this sequence and the template was identical to that used previously (Lamb 1990).

8.2 Analysis of the immediate promoter

This region was selected for further study as this was found to be a DHS, in addition very often many genes contain important regulatory regions within the first 200bp of the transcriptional start site. As the purpose of the project was to identify sequences which confer myeloid-specific and differentiation stage-specific expression, DNase1 foot printing of the region -176 to +15 was undertaken using HL60 nuclear extract. As this cell line does not express the defensin genes this is not the best choice of extract, but was employed for the following reasons. Primarily, the lack of availability of CML samples of a size to generate the amount of nuclear protein necessary for DNAse1 foot printing studies ruled out their use in this study. In addition, the heterogeneity of these samples would also pose a problem in the interpretation of the results, because it would not allow the identification of myeloid-

-650 GATGATCCTA GACAATTGTT TAACCTTAAA CTGTTCATTG GCCAAGCAAA

-601 CAGGGTGATA GTCACCTCTG GGGAACCACA TGCCGCGTGT ACATCCAGTA

-551 CTCAGGAGAA CCCAAAAATG TCTGTTCCAC ATAGCAACAG AAGCCCAGGT

-501 AGCACTCAGT CTCTCCTGGG TGTTCTCCAA CATCCCAGCT CACCAAATGG

-450 CTTTCATTAG TTTTTATGGT TAGAACCCCA GGTCCTCGGG ACACTGCTTT

-400 AGAAACACAT TCCAAATCCT CCTCTGTGTG CAGGTGGCAA TCCTATCCCA

-350 ATCTCTTTGC AGGGTGTATA CTATGATACG CAGCCAGGCT GTCCCAGAGG

-300 CCTTAAATAT TTCCTTGGTG CAGGCAGTTC AGCTTAGCCA CAGCCAATGC

MBS

-250 ATCACAGGCT CAACTGTGTT AGGAGCCATT GAGAATCCAT AGTTGGTTGC

-200 TGCCTGGGCC TGGCCAGGGC TGACCAAGGT AGATGAGAGG TTCCTCTGTG

MBS

-150 GAGTTCTACT TTAACCTCAC CTTCCCACCA AATTTCTCAA CTGTCCTTGC

C

-100 CACCACAATT **ATTTA**ATGGA CCCAACAGAA AGTAACCCCG GAAATTAGGA

-50 CACCTCATCC CAAAAGACCT T**TAAATA**GGG GAAGTCCACT TGTGCACGGC

TGCTCCTTGC TATAGAA

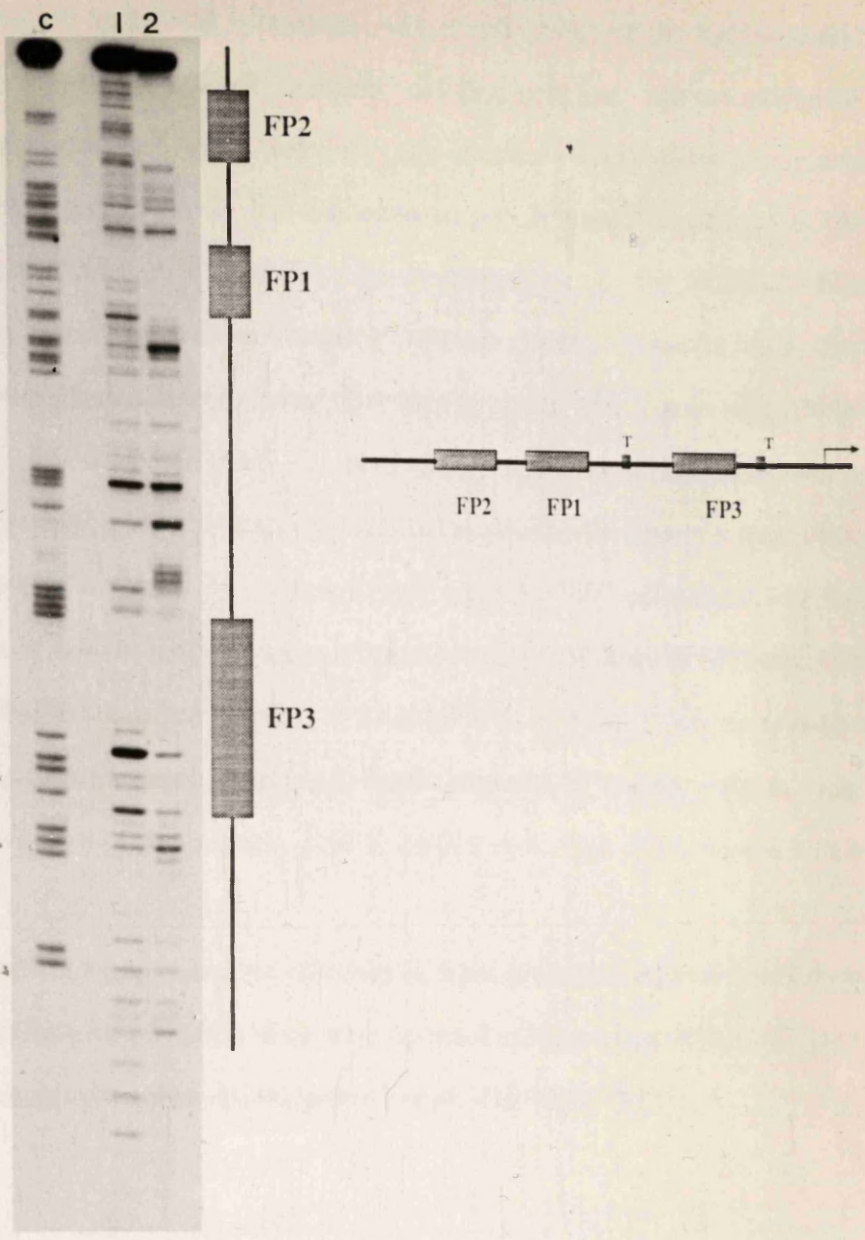
Figure 8.1: Upstream sequence of the defensin HNP1A genomic clone. The numbers are relative to the major site of transcriptional initiation. The TATA box homologies are shown in bold, and the putative c-Myb binding sites are labelled MBS. The nucleotide at position -94 shows a discrepancy between this sequence and the one previously determined (shown above the sequence)

Figure 8.2 A: DNAse1 footprinting of the region -176 to +15 of the defensin HNP1A promoter.

Footprinting of the promoter fragment -176 to +15 as described in methods. Lane C, Maxam and Gilbert C track; lane 1, probe incubated in the absence of nuclear extract; lane 2, probe incubated with 200µg HL60 nuclear extract. The protected regions are represented by the shaded boxes. The footprints identified are labelled FP1, FP2 and FP3. The positions of these footprints are illustrated on the cartoon of the promoter, T=TATA box.

B: Sequence of the promoter illustrating regions of the probe showing protected/enhanced cleavage.

A



B

```
-233 GTTAGGAGCC ATTGAGAATC CATAGTTGGT TGCTGCCTGG GCCTGGCCAG
        XX XX
     GGCTGACCAA GGTAGATGAG AGGTTCCTCT GTGGAGTTCT ACTTTAACCT
                                  XO OOX
     CACCTTCCCA CCAAATTTCT CAACTGTCCT TGCCACCACA ATTATTTAAT
           XX XX
     GGACCCAACA GAAAGTAACC CCGGAAATTA GGACACCTCA TCCCAAAAGA
-33  CCTTTAAATA GGGGAAGTCC ACTTGTGCAC GGCTGCTCCT TGCTATAGAA
```

X Enhanced cleavage

O Neither protection nor enhanced cleavage

Footprints

specific transcription factors these samples contain cells from all the haemopoietic lineages. Also the cells in the CML samples, unlike a cell line, are not primarily arrested at a specific stage of differentiation, but contain a range of immature and mature leukocytes. Use of a cell line was necessary as it would be limited to the myeloid lineage and arrested at a specific stage of differentiation. The HL60 cell line was chosen as it had in the past expressed the defensin genes so it seems likely that many of the transcription factors necessary for the expression of this gene will still be present in these cells. It seems likely that the lack of expression of the defensins is the result of either methylation of important control regions or the loss of a single key transcription factor (section 6.6), which would suggest HL60 cells would be the next best choice of extract in the absence of an expressing cell line. In addition, the use of this cell line would allow changes in a model of human myelopoiesis assessed using controlled experimental conditions. Small amounts of nuclear extract from CML samples was available and was used in EMSA described in sections 8.3, 8.4 and 8.5.

This footprinting revealed the presence of three protected regions (see figure 8.2), the precise locations of these sites were determined by comparison to Maxam and Gilbert sequencing reactions of the probe lanes C G+A and T+C.

FP1 -124 to -101

FP2 -175 to -151

FP3 -70 to -46

8.3 Investigation of the FP1 DNA binding activities

After finding the region spanning -124 to -101 was protected by HL60 nuclear extract in DNAse1 foot printing studies, electrophoretic mobility shift assays (EMSA) were employed as a tool to address a number of questions. These were:

1) What is the tissue distribution of this activity i.e. is it myeloid-specific?

2) Does this DNA binding activity change during differentiation?

3) What are the transcription factor(s) which comprise this activity?

8.3 (i) Tissue distribution of the FP1 DNA binding activity

The use of the EMSA technique offers a number of advantages over footprinting; it allows factors which bind the same sequence to be distinguished by virtue of the relative mobility's of the DNA-protein complexes, smaller amounts of nuclear protein are required and the technique is more sensitive than footprinting. Complementary oligonucleotides spanning the protected region of the footprint were generated, annealed and radio labelled as described in section 4.17. A series of nuclear extracts from a range of cell lines were tested for FP1 DNA binding activity (see figure 8.3A). This result demonstrates that an HL60 nuclear extract generates a retarded complex which, on close analysis is found to be comprised of a doublet of equal intensity. The nuclear extract from a CML patient which contained 30% myelocytes had no measurable FP1 binding activity. This lack of activity probably reflects an absence of FP1 binding proteins in this sample as the integrity of all samples were verified by analysis with other labelled oligonucleotides, although minor variations in the quality of samples cannot be discounted. Nuclear proteins derived from the circulating leukocytes of a patient with neutrophilia were used as source of neutrophils (the sample contained 90% neutrophils) and the retarded complex generated can be seen in lane 4. This is a surprising result as this FP1 binding activity is lost on granulocytic differentiation of HL60 cells (see section 8.3 (ii)), however the complex has a different mobility and may be induced by an inflammatory reaction which may occur in neutrophilias. The activity of C/EBP transcription factors can be modulated in response to inflammation, notably C/EBPβ and C/EBPδ and the FP1 binding activities may be comprised of transcription factor(s) from this family (see section 8.3 (iii)). All the other extracts tested generated a complex of similar mobility to that generated with HL60 extracts, as well as a number of other complexes from

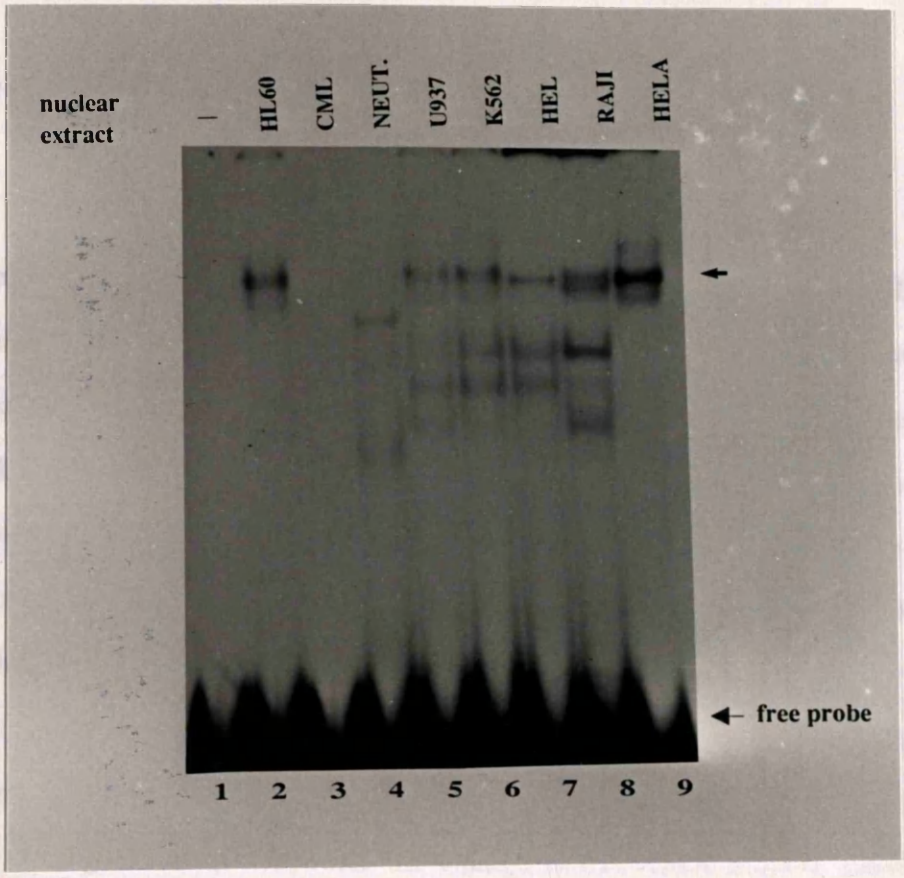


Figure 8.3: EMSA of FP1 radiolabelled probe, using extracts from a range of cell lines and patient samples.

The left hand lane (1), contains probe in the absence of nuclear protein. Each of the other lanes contains 10μg of nuclear extract from the following cell line/sample; HL60, chronic myeloid leukaemia leukocytes, neutrophils derived from a neutrophila patient, U937, K562, HEL, Raji, Hela.

both other myeloid cell lines and non-myeloid cell lines including the epithelial cell line HeLa.

8.3 (ii) Effect of differentiation on FP1 binding activities

Given that the CML sample had no FP1 DNA binding activity, it was of interest to determine if this activity was regulated during differentiation. To test this possibility nuclear extracts obtained from a time course of HL60 TPA and DMSO induced differentiation were assessed for their ability to bind labelled FP1 probe in an EMSA. The differentiation was assessed during the DMSO induction using NBT staining, and by morphological examination during the TPA induction. These nuclear extracts were used for all of the following experiments to allow direct comparison between different probes eliminating concern that differences could be due to the differences in the quality of extracts between inductions / variations in other conditions (although the inductions were repeated).

Figure 8.4 B illustrates the change in FP1 DNA binding activity during granulocytic differentiation as measured by EMSA. Interestingly the levels of FP1 DNA binding activity decrease such that there is little or no activity remaining 8 hours post DMSO induction, suggesting that loss of this activity is a rapid event following the induction of granulocytic differentiation of HL60 cells. In contrast, during TPA induced differentiation (figure 8.4 A) a rapid transient increase in this activity occurs before a gradual decrease in the fully differentiated cell to levels similar to those in uninduced cells. These results suggested that this factor(s) is regulated in a differentiation-stage-specific manner which is dependent on the pathway of differentiation.

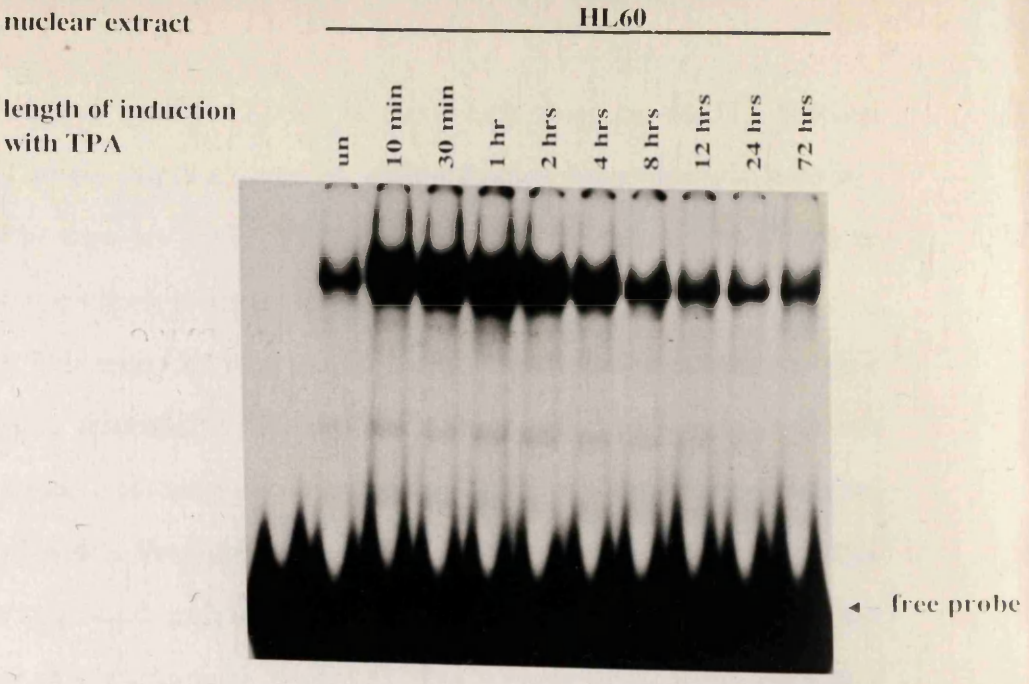
Figure 8.4 : EMSA showing changes in the FP1 binding activity during HL60 differentiation.

HL60 cultures were induced to differentiate using DMSO and TPA and nuclear protein was harvested at a range of time points as described in section 8.3(ii). These samples were used in the EMSA illustrated in this figure. With the exception of the left hand lane, each reaction contained 10μg of nuclear protein. un=uninduced HL60 nuclear extract, each of the other labelled lanes refers to the length of time post induction that the nuclear extract was harvested. Free probe can be seen at the bottom of the gel.

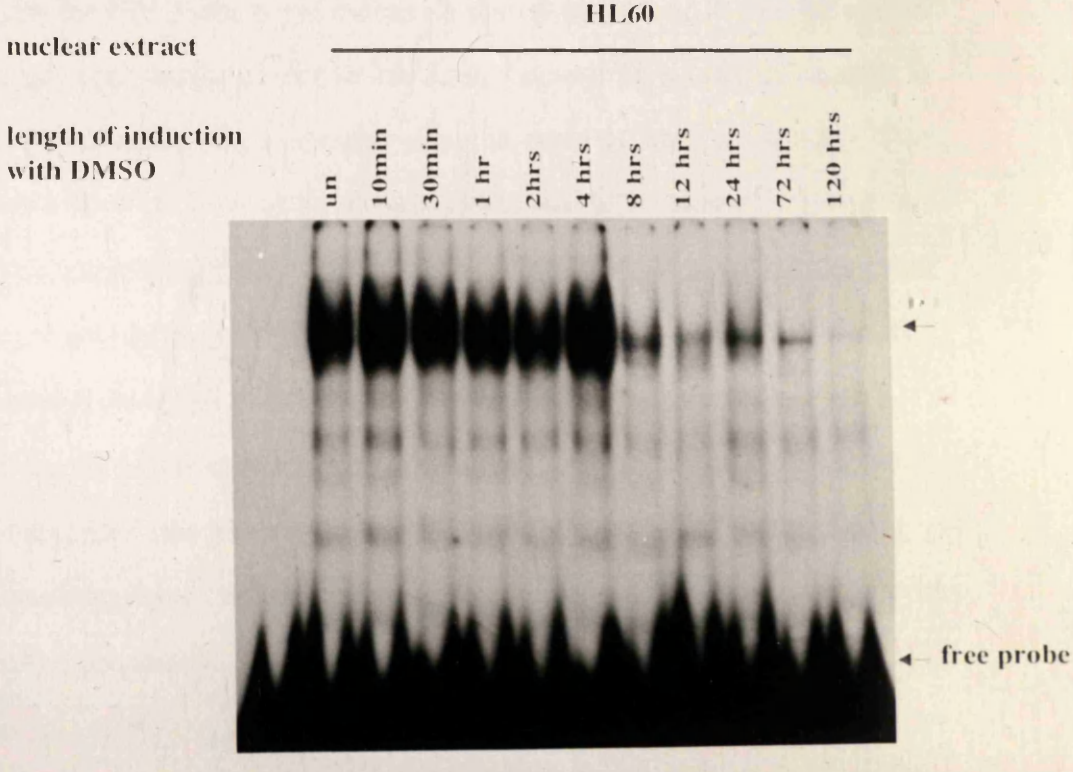
A: TPA induction. The differentiation induction was monitored by morphological examination. Over 90% of the cells became adherent within 24 hours.

B: DMSO induction. The differentiation induction was monitored by NBT staining. uninduced cells 5%, 24 hours 10%, 72 hours 45% and 120 hours 82% of cells were NBT positive.

A


nuclear extract
HL60
length of induction with TPA
un
10 min
30 min
1 hr
2 hrs
4 hrs
8 hrs
12 hrs
24 hrs
72 hrs
free probe

B


nuclear extract
HL60
length of induction with DMSO
un
10min
30min
1 hr
2hrs
4 hrs
8 hrs
12 hrs
24 hrs
72 hrs
120 hrs
free probe

8.3 (iii) Characterisation of the FP1 DNA binding activity

The identity of the transcription factors which comprise the FP1 binding activity was of prime interest given its differentiation stage-specific activity. Examination of the sequence of the FP1 oligonucleotide offered a number of clues as to possible binding proteins, most notably the presence of a potential c-Myb binding site (figure 8.1). This sequence is an almost perfect match for the extended c-Myb consensus sequence generated by Watson *et al.*, (1991) using a PCR based selection method. In addition, a potential binding site for C/EBP was also present. To gain further information as to the nature of these FP1 DNA binding activities an EMSA with FP1 probe and HL60 nuclear extract was carried out in the presence of excess cold competitor oligonucleotides known to bind a range of transcription factor families (figure 8.5 A). This revealed that a symmetric C/EBP binding sequence could compete out the FP1 binding complexes. Some competition was also seen using FP3 oligonucleotide, the reason for which is discussed in section 8.5 (iii). Despite the presence of a perfect c-Myb binding site, no competition was seen using 100-fold excess of cold c-Myb binding oligonucleotide, suggesting that the complex generated with the FP1 probe is not the result of c-Myb binding. A role for c-Myb binding this sequence *in vivo* cannot be ruled out, because no binding of proteins to a consensus Myb binding oligonucleotide could be detected (data not shown). This suggests that c-Myb is either at an abundance too low to be identified using this technique, or alternatively the conditions employed in this assay are not those required for c-Myb binding *in vitro*. Further investigation of the role of c-Myb acting at this sequence is discussed in chapter 9.

The results of the competition experiment suggested that the FP1 binding activities were C/EBP-like (or rather were capable of binding C/EBP binding sites) and a further experiment was undertaken to assess if these activities had another of the C/EBP family's properties. The C/EBP family are heat stable (Graves *et al.*, 1986; Johnson *et al.*, 1987), and an EMSA was undertaken after HL60 nuclear

Figure 8.5 A: Analysis of the DNA binding properties of the FP1 binding activities by EMSA.

With the exception of lane 1, each reaction contains 10μg of HL60 nuclear extract incubated with radiolabelled FP1 probe. Competitor oligonucleotides were included (as indicated in the figure) at 100 fold molar excess. The retarded complex I is indicated by the arrow, fp=free probe.

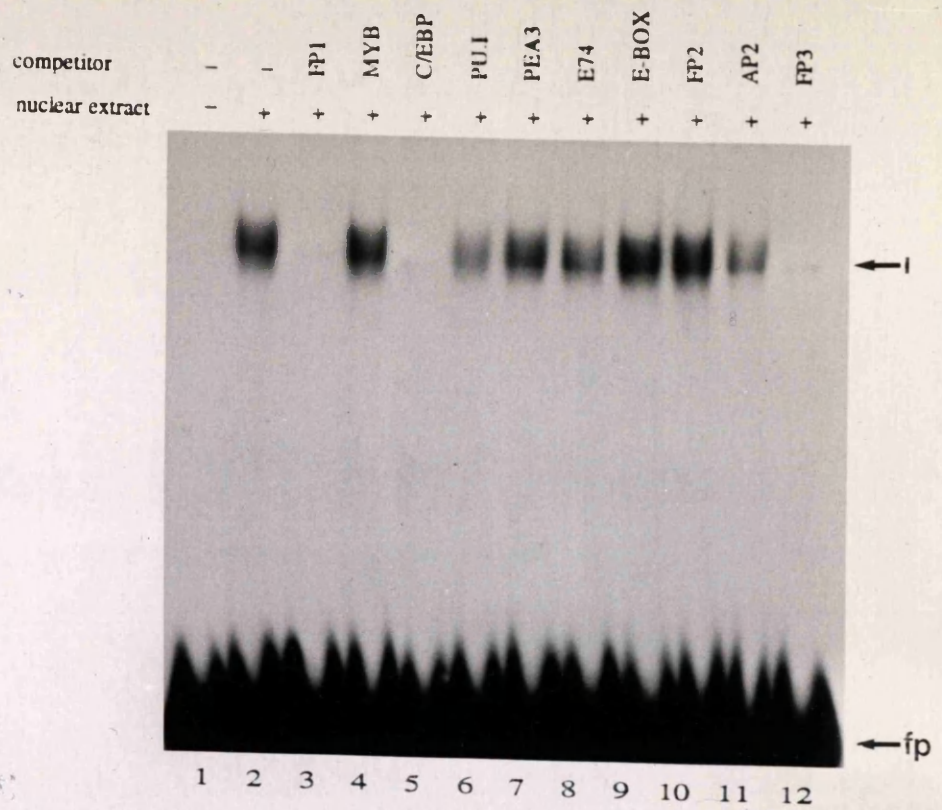
The sequence of the sense strand of the competitor oligonucleotides are listed

FP1: GGGTTCCCACCAAATTTCTCAACTGTCCTGCCGG
FP2: GGTAGATGAGAGGTTCCTCTGTGGAGTTCTACTTTAA
FP3: ACAGAAAGTAACCCCGGAAATTAGGACACCTCATCCC
AP2: CCGGCCCCAGGCGT (Oka *et al.*, 1991)
MYB: TTCGGCATAACGGTTCCGTAGCC (Watson *et al.*, 1992)
C/EBP: GCTGCAGATTGCGCAATCTGCAGC (Grove and Plumb 1994)
PU.1: CGTCCCAAGAGAGGAACCAATCAGCATTG (Klemz *et al.*, 1990)
E74: AGCTTCTCTAGCTGAATAACCCGGAAAGTAACTCATCGTCGA (Urness and Thummel 1990)
PEA3: GATCGAGGAAGTGAGTGAGTAACG (Martin *et al.*, 1988)

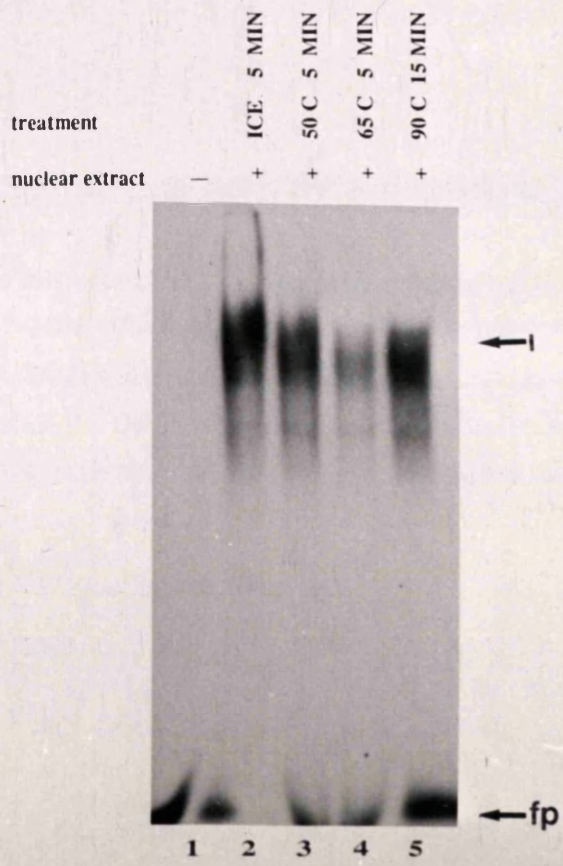
Figure 8.5 B: Analysis of the heat stability of the FP1 binding activities by EMSA with radiolabelled FP1 probe.

An EMSA was carried out using FP1 radiolabelled probe. With the exception of lane 1 each reaction contained 10μg of HL60 nuclear extract given a prior heat treatment. The lengths and temperatures of these treatments are indicated in the figure. The retarded complex is labelled I and the free probe can be seen at the bottom of the gel

A


competitor
nuclear extract
–
–
+
FP1
MYB
C/EBP
PU.1
PEA3
E74
E-BOX
FP2
AP2
FP3
I
fp
1 2 3 4 5 6 7 8 9 10 11 12

B


treatment
nuclear extract
–
ICE 5 MIN
50 C 5 MIN
65 C 5 MIN
90 C 15 MIN
+
I
fp
1 2 3 4 5

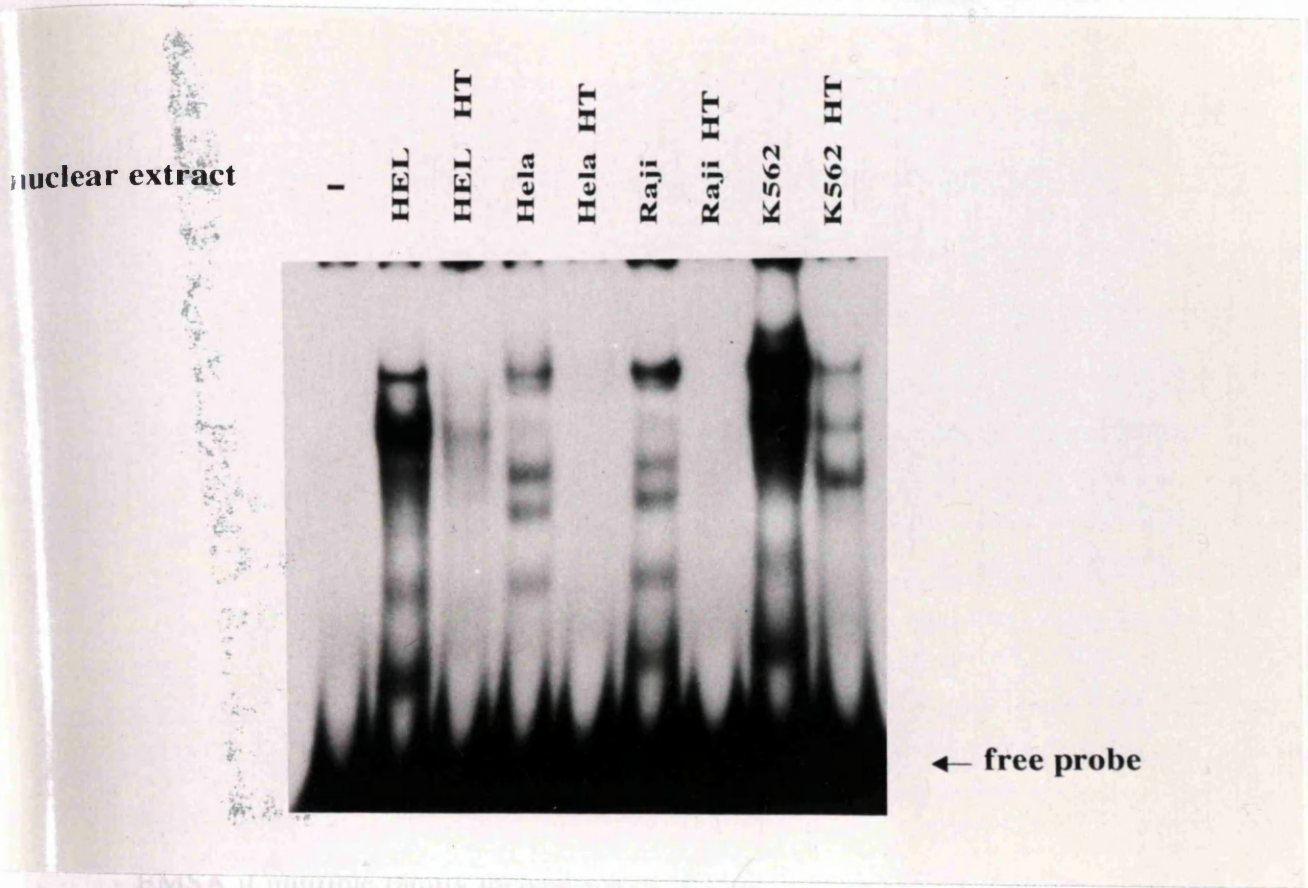


Figure 8.6: Distrubution of heat stable FP1 binding activity as measured by EMSA

The distribution of heat stable FP1 activity was tested using EMSA with radiolabelled FP1 probe. With the exception of lane 1, each lane contained nuclear protein (5μg) from the cell line indicated in the figure. The lanes indicated by HT correspond to reactions set up after the nuclear protein was heated to 90°C for 15 minutes. Free probe can be seen at the bottom of the gel. N.B. The K562 sample contained 10μg of nuclear protein.

extract was given a range of heat treatments (figure 8.5 B). This revealed that the FP1 binding activities are heat stable, even to 90°C for 15 minutes, suggesting that the FP1 binding activity(s) are C/EBP-like.

A question still to be answered was, are the FP1 binding activities in other cells the same/similar to those characterised in the HL60 cell line? This was a particularly interesting question given the reports that within the haemopoietic system the C/EBP family of transcription factors are limited to the myeloid lineages. A range of extracts were similarly heat treated to test if the FP1 binding activities in these cell lines were also heat stable. As can be seen in figure 8.6, after heat treatment of nuclear extracts for 15 minutes at 90°C, FP1 binding activity in HeLa and Raji was abolished. K562 shows some activity after heat treatment but a reduction in activity is apparent. Whether these represent C/EBP-like activities is not clear, but as this cell line is not strictly erythroid showing some myeloid characteristics (Marie *et al.*, 1981), expression of C/EBP transcription factors would perhaps not be surprising.

The C/EBP family comprises a range of members (section 3.4) capable of forming homo and heterodimers, these different dimers generate proteins of different molecular weights and would be expected to generate a range of complexes in a EMSA if multiple family members were present. This is not the case for the HL60 extract which has a single major complex (although this is comprised of a doublet). To test if this sequence was only binding a subset of C/EBP dimers, a series of EMSA were undertaken with a consensus C/EBP binding site used as the probe.

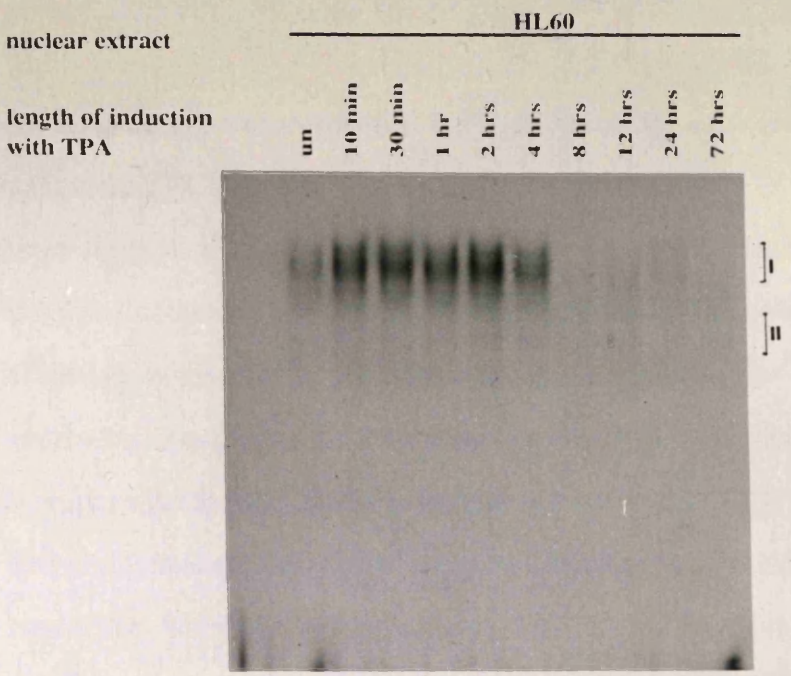
The C/EBP binding activities were assessed during HL60 differentiation by EMSA using aliquots of the same nuclear extracts described in section 8.3 (ii). The EMSA of C/EBP binding activity during HL60 differentiation can be seen in figure 8.7. Comparison of C/EBP and FP1 binding activities during HL60 differentiation suggest they are very similar (cf. figures 8.4 and 8.7). Closer examination however, reveals a number of apparent differences. Considering first the DMSO induction (figure 8.7 B). This reveals that complex I decreases during differentiation; however, the loss of this binding activity is less precipitous than that of the FP1 binding activities

Figure 8.7 : EMSA showing changes in C/EBP binding activity during HL60 differentiation.

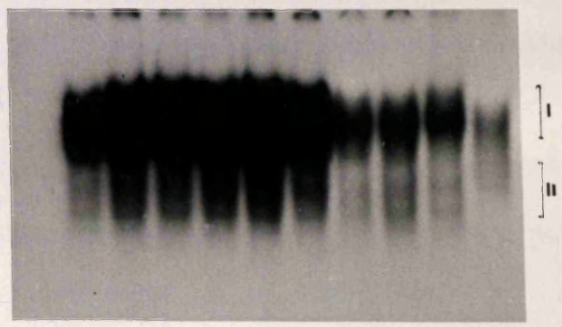
HL60 cultures were induced to differentiate using DMSO and TPA and nuclear protein was harvested at a range of time points as described in section 8.3(ii). These samples were used in the EMSA illustrated in this figure. With the exception of the left hand lane, each reaction contained 10μg of nuclear protein. un=uninduced HL60 nuclear extract, each of the other labelled lanes refers to the length of time post induction that the nuclear extract was harvested. Free probe can be seen at the bottom of the gel. Retarded complexes referred to in the figure are labelled I and II.

Ai: TPA induction.
Aii: longer exposure of the complexes seen in Ai.
B: DMSO induction.

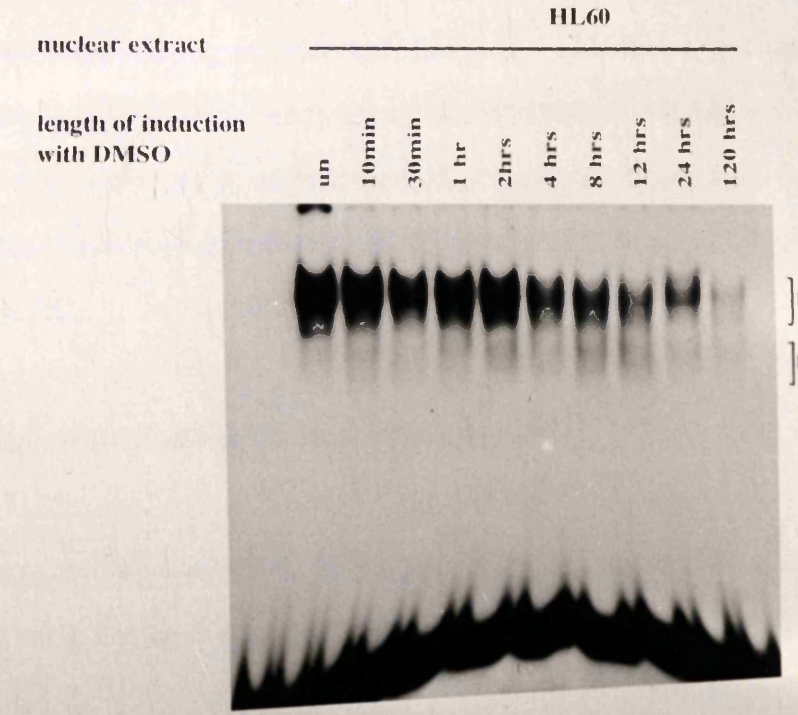
Ai


nuclear extract
HL60
length of induction with TPA
un
10 min
30 min
1 hr
2 hrs
4 hrs
8 hrs
12 hrs
24 hrs
72 hrs
I
II

Aii


I
II

B


nuclear extract
HL60
length of induction with DMSO
un
10min
30min
1 hr
2hrs
4 hrs
8 hrs
12 hrs
24 hrs
120 hrs
I
II
free probe

(figure 8.4). Another difference is the presence of the higher mobility complex II, which is not generated when FP1 is used as a probe. Changes in the intensity of this complex do not mirror those of the major complex, perhaps suggesting they are generated by different transcription factors. Whether these higher mobility complexes represent factors at low abundance, or factors which have a low affinity for this oligonucleotide is unknown. Considering the TPA induction (figure 8.7 A), again a superficially similar pattern is obtained to that generated when using a FP1 probe, but there are a number of differences. The level of complex I in the uninduced extract are at least 5-fold greater than that in the fully differentiated cell. This is in contrast to the FP1 binding activities which are at similar levels in both uninduced and fully differentiated cells. The complex II seems to mirror changes in complex I during this induction.

A possible explanation of these results is that the FP1 oligonucleotide binds only a subset of C/EBP dimers present in these nuclear extracts, and the pattern generated using the C/EBP probe is the result of a number of different dimers binding. This could explain why the rate of loss of the C/EBP binding activity is slower than that of the FP1 binding activity during granulocytic differentiation. If dimers which are differentially regulated, generated retarded complexes of similar mobility then the result of the EMSA using the C/EBP probe may represent a composite picture of the binding of a number of factors. Evidence supporting this proposition can be seen by close examination of the TPA induction (figure 8.7Aii) which shows that the complex I is composed of a number of bands of similar mobility. Complex II may represent dimers (or a degradation product of complex I) which do not bind FP1.

8.4 Investigation of the FP2 DNA binding activities

Similarly to the approach taken to characterise FP1, a number the questions where addressed using EMSA with a double-stranded oligonucleotide probe FP2

8.4 (i) Tissue distribution of the FP2 DNA binding activities

Nuclear extracts from a range of cell lines were assessed for FP2 binding activities using EMSA (see figure 8.8). Specific complexes are labelled I and II. 'Non-specific' complexes cannot be competed by the addition of excess cold 'self' oligonucleotide to the reaction and hence do not correspond to sequence-specific DNA binding proteins. Two complexes of similar mobility are generated by nuclear extracts derived from the HL60 cell line and the other myeloid cell lines (although very faint in the U937 extract). In addition, these two complexes are also generated by extracts derived from the pre-B cell line Raji. These complexes, however, seem to be absent from the reaction containing the HeLa cell extract, suggesting that perhaps these complexes are generated by transcription factors which are restricted to the haemopoietic compartment. Clearly, however, it is not possible to determine if this is the case without a more widespread screen using nuclear extract from a number tissues.

8.4 (ii) Effect of differentiation on FP2 DNA binding activities

EMSA was employed to measure any changes in FP2 DNA binding activity during HL60 differentiation (nuclear extracts were those described in section 8.3 (ii)). Figure 8.9 B illustrating DMSO-induced granulocytic differentiation, demonstrates that the two specific complexes I and II remain constant during differentiation down this pathway. During TPA-induced differentiation, however, (figure 8.9 A) a small increase in FP2 DNA binding activity is apparent. A transient increase in the expression of many genes is seen on TPA treatment of HL60 cells (e.g. *myc*, *max*, ornithine decarboxylase), and may represent a TPA response which is separable from the induction of the differentiation program, particularly as in this case the response is relatively small. Other than this small increase the levels of these two complexes remain unaltered during HL60 differentiation.

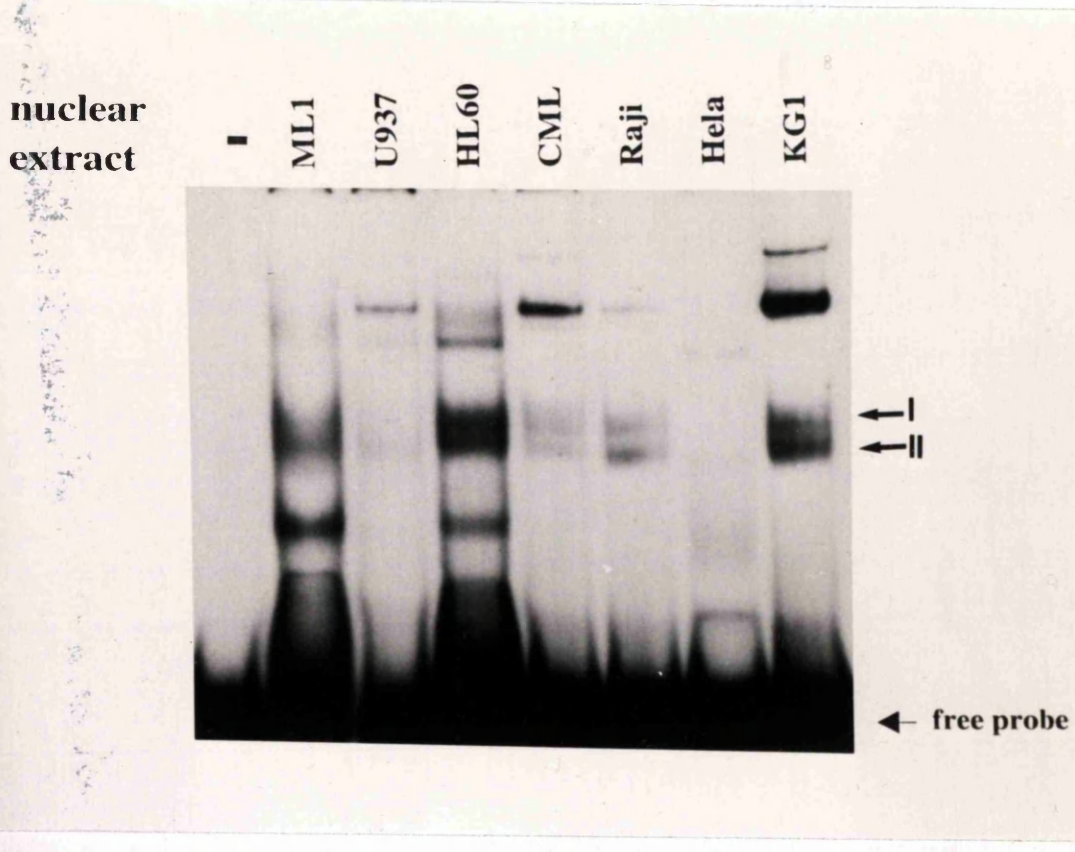


Figure 8.8: EMSA of FP2 radiolabelled probe, using extracts from a range of cell lines and patient samples.

Lane 1 contains probe in the absence of nuclear protein. Each of the other lanes contains 10μg of nuclear extract derived from the cell line/patient sample indicated in the figure. Retarded complexes I and II are refered to in the text, free probe can be seen at thebottom of the gel.

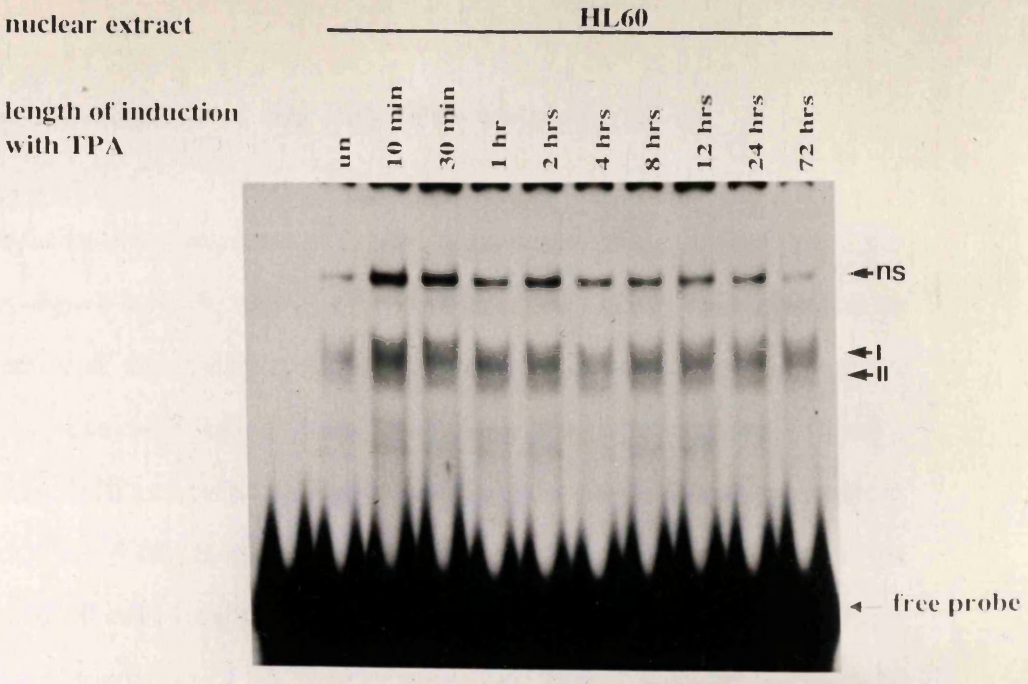
Figure 8.9 : EMSA showing no changes in the FP2 binding activity during HL60 differentiation.

HL60 cultures were induced to differentiate using DMSO and TPA and nuclear protein was harvested at a range of time points as described in section 8.3(ii). These samples were used in the EMSA illustrated in this figure. With the exception of the left hand lane, each reaction contained 10µg of nuclear protein. un=uninduced HL60 nuclear extract, each of the other labelled lanes refers to the length of time post induction that the nuclear extract was harvested. Specific retarded complexes are labelled I and II, ns=non-specific. Free probe can be seen at the bottom of the gel.

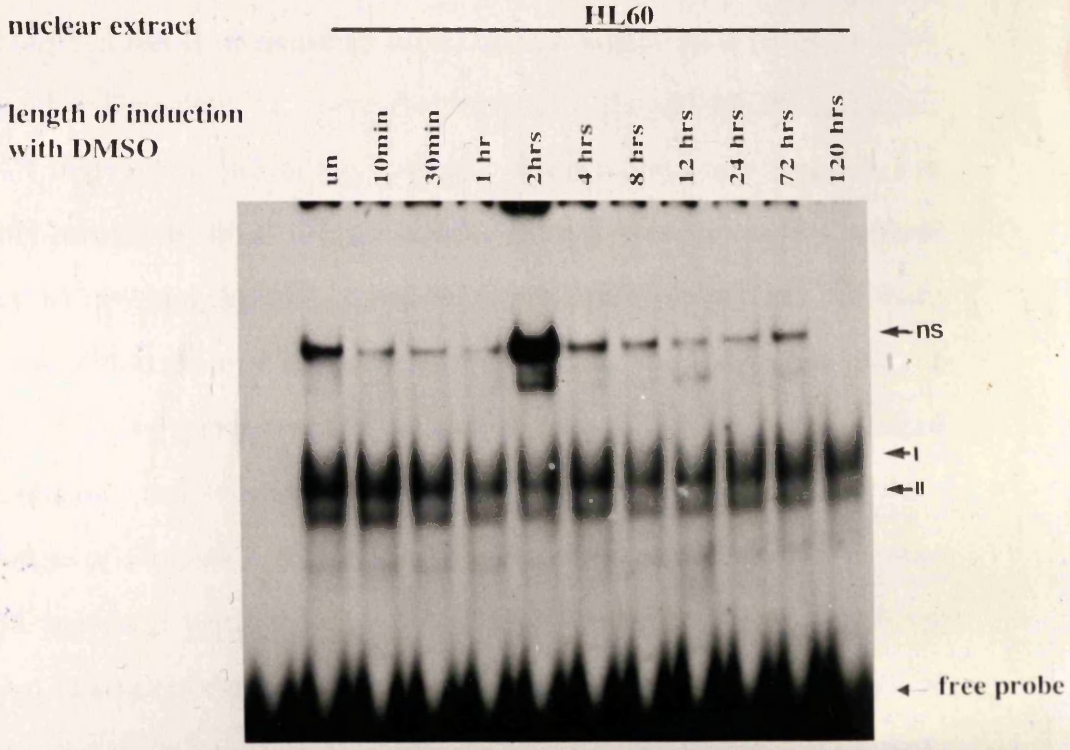
A: TPA induction.

B: DMSO induction.

A


nuclear extract
HL60
length of induction with TPA
un
10 min
30 min
1 hr
2 hrs
4 hrs
8 hrs
12 hrs
24 hrs
72 hrs
ns
I
II
free probe

B


nuclear extract
HL60
length of induction with DMSO
un
10min
30min
1 hr
2hrs
4 hrs
8 hrs
12 hrs
24 hrs
72 hrs
120 hrs
ns
I
II
free probe

8.4 (iii) Characterisation of the FP2 DNA binding activity

Examination of the sequence of the FP2 sequence revealed a GGAA sequence or an Ets box (figure 8.10 B), which is the core homology of the binding sequence of the Ets family of transcription factors. Flanking sequences determine which particular factors bind with high affinity. Comparison of the FP2 sequence to known Ets binding sites (EBS) revealed the highest similarity to the PU.I binding site from the SV40 promoter (Klemsz *et al.*, 1990; figure 8.10 B). To test if the complexes present in the HL60 cells had Ets-like binding properties competition EMSA were undertaken using a number of Ets binding and other oligonucleotides (figure 8.10 A).

Cold excess of oligonucleotides FP3, FP1, PU.I and E74 can compete out FP2 binding activity. With the exception of FP1, all of these oligonucleotides have clear similarity to EBS (figure 8.10 B). Although FP1 does not contain a perfect Ets box, it does contain two sequences which have sequence similarity to EBS. The two FP2-binding complexes I and II generated by HL60 nuclear extract show different DNA binding properties. The complex II can be competed out by the addition of excess cold PU.I and E74 oligonucleotides to the reaction, whereas the upper complex I is competed only partially by these oligonucleotides. Both complexes can be competed with FP1 and FP3 oligonucleotides. There are two possible explanations for these results; the two complexes may be generated by distinct DNA binding proteins, or may be two forms of the same protein. If the latter is correct this could be the result of alternative splicing, post-translational modification, or the complex II may be a cleavage product of complex I. As previously mentioned a number of ETS proteins contain PEST sequences (section 3.5), which make them particularly susceptible to cleavage. Also cleavage of the Ets proteins can alter their DNA binding properties, as limited proteolysis of Ets-1 increasing its affinity for the PEA3 sequence (Lim *et al.*, 1992). If the lower complex lost similar sequences it is possible that its

Figure 8.10 Ai: Analysis of the DNA binding properties of the FP2 DNA binding activities using EMSA.

Each lane with the exception of lane 1 contains 10μg of HL60 nuclear protein. Unlabelled competitor oligonucleotides were included (as indicated in the figure) at 100 fold molar excess. Arrows indicate retarded DNA-protein complexes I and II.

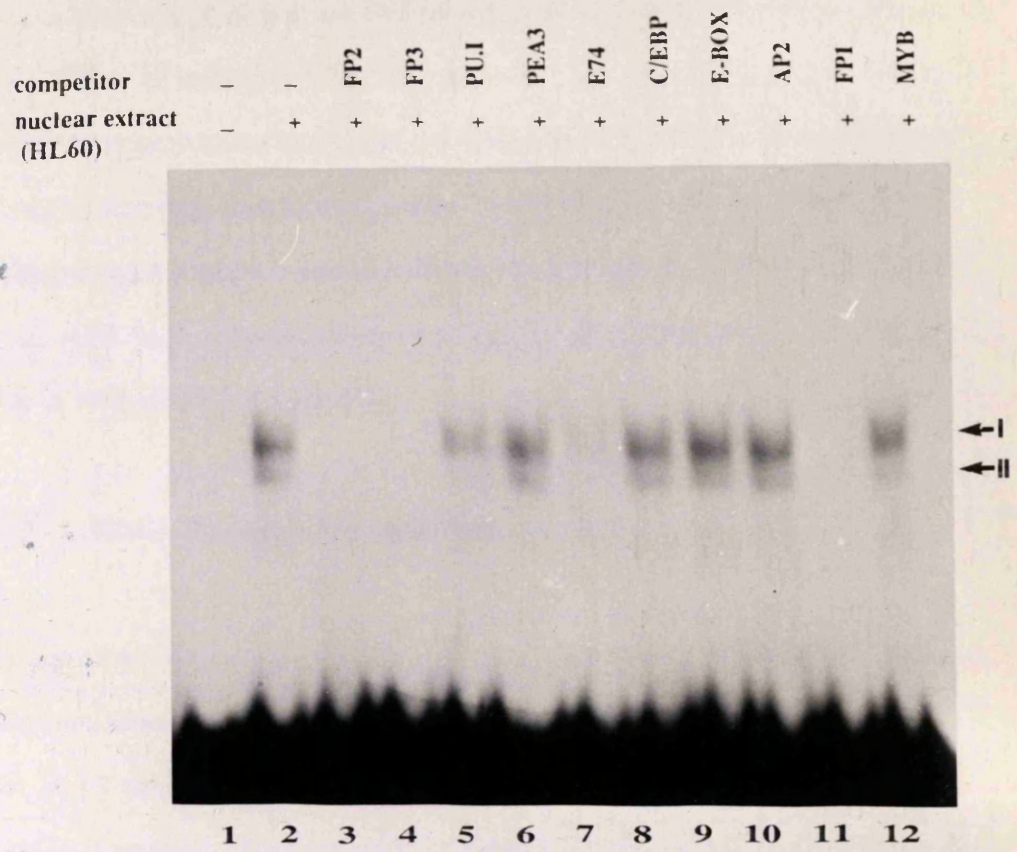
The sequence of the sense strand of the competitor oligonucleotides are listed

FP1: GGGTTCCCACCAAATTTCTCAACTGTCCTGCCGG
FP2: GGTAGATGAGAGGTTCCTCTGTGGAGTTCTACTTTAA
FP3: ACAGAAAGTAACCCCGGAAATTAGGACACCTCATCCC
AP2: CCGGCCCCAGGCGT (Oka *et al.*, 1991)
MYB: TTCGGCATAACGGTTCCGTAGCC (Watson *et al.*, 1992)
C/EBP: GCTGCAGATTGCGCAATCTGCAGC (Grove and Plumb 1994)
PU.1: CGTCCCAAGAGAGGAACCAATCAGCATTG (Klemz *et al.*, 1990)
E74: AGCTTCTCTAGCTGAATAACCCGGAAAGTAACTCATCGTCGA (Urness and Thummel 1990)
PEA3: GATCGAGGAAGTGAGTGAGTAACG (Martin *et al.*, 1988)

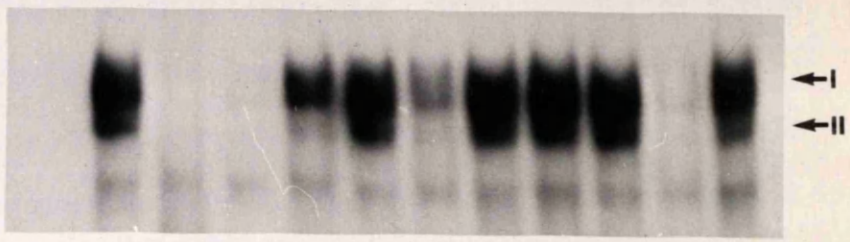
Aii: Longer exposure of the complexes present in Ai

B: Diagram illustrating the Ets binding sequences used in this study.

Ai



Aii



B

PEA3	CGAGGAAGTG	Wasylyk et al. (1990)
E74A	CCCGGAAGTA	Urness and Thummel (1990)
PU.I	AGAGGAACTT	Klemsz et al (1990)
NF-JB	AGCAGAAGCA	Shin and Koshland (1993)
FP2	AGAGGAACCT	
FP3	CCCGGAAATT	
FP1	CAAGGACTTG	
	TGAGAAATTT	

DNA binding properties would be altered and could explain its ability to be competed by a wider range of EBS.

The evidence suggests that the FP2 binding proteins are Ets family members, however, a number of known Ets family members may comprise these activities. These complexes appear to be limited to the haemopoietic compartment (although they could not be detected in erythroid cells) suggesting that possibly PU.I (Spi-1) or Spi-B which have a limited tissue distribution and would be predicted to bind to this sequence with high affinity (Klemsz *et al.*, 1993; Galson *et al.*, 1993) may generate one or both of these complexes.

8.4 (iv) Spi-1 binds to the FP2 sequence

The transcription factor PU.1 was a strong candidate to comprise the FP2 DNA binding activities as: this factor is restricted to the haemopoietic compartment, it binds the PU.1 sequence, it generates a high mobility complex and in some nuclear extracts it produces two complexes of similar mobility as result of cleavage at the PEST sequence (Pahl *et al.*, 1992; Galson *et al.*, 1993). To determine if PU.1 could bind the FP2 sequence, Spi-1was generated *in vitro* to use in an EMSA (Spi-1 is the mouse homologue of PU.1; the proteins are 98% identical at the amino acid level). Spi-1 RNA was generated by *in vitro* transcription of the full length cDNA and this was then translated using a reticulocyte lysate as described in section 4.18. A duplicate translation was carried out which was labelled by the addition of ^{35}S-methionine. A portion of this translation was resolved on SDS-PAGE (section 4.21 ii). Autoradiography revealed that the majority of the label was incorporated in a single product of approximately 40 kDa, which is the size expected for a full length translation of Spi-1 (Zhang *et al.*, 1993). An EMSA was then carried out using radiolabelled FP2 as a probe and the unlabelled Spi-1 programmed reticulocyte lysate. This experiment revealed that Spi-1 could bind to this sequence (figure 8.11 A). The Spi-1-specific complexes are labelled SI and SII in the figure. These

complexes can be eliminated by inclusion of excess cold competitor FP2 oligonucleotide but not FP1, indicating that the later has an affinity for this factor which is lower than that of FP2.

As Spi-1 could bind the FP2 sequence, the mobility of the FP2 binding complexes generated by HL60 nuclear protein and the Spi-1 primed reticulocyte lysate were compared by EMSA. The results of this experiment can be seen in figure 8.11B, illustrating that the major complex generated using the Spi-1 reticulocyte lysate SI, has a very similar mobility as the HL60 FP2 binding complex I. This evidence lends weight to the proposition that the FP2 binding activity I is the PU.1 transcription factor. Whether the lower complex represents PU.1 after cleavage at the PEST sequence is unknown.

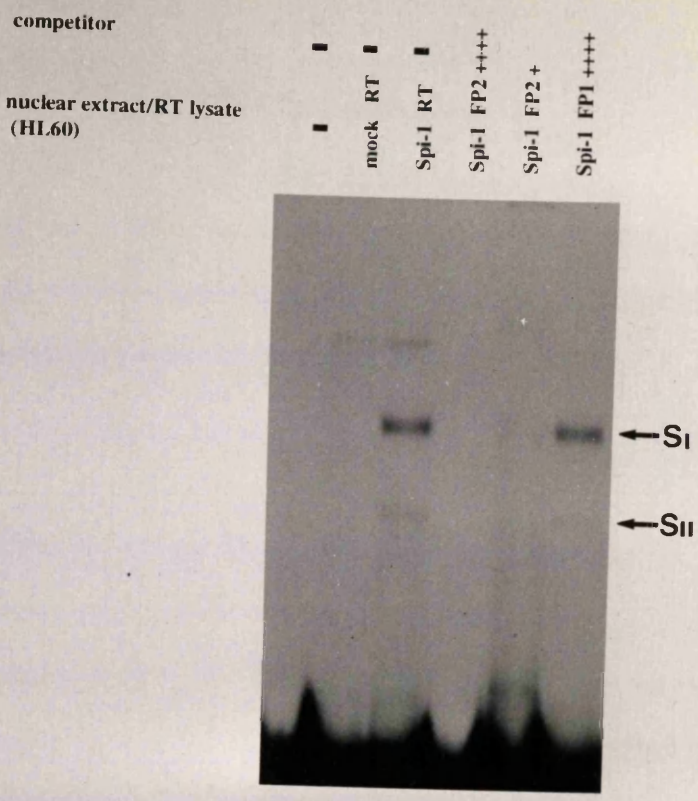
Figure 8.11 EMSA showing Spi-1 binds the FP2 sequence.

A: EMSA using Spi-1 reticulocyte lysate and FP2 radiolabelled probe. To test if the Spi-1 transcription factor could bind to the FP2 sequence, *in vitro* transcription/translation of a Spi-1 template (Nibbs 1993) using reticulocyte lysate (as described in section 5 of methods) was employed in a EMSA. Lane 1 probe alone; 2, 10µl mock RT lysate; 3, 10µl Spi-1 RT lysate; 4, 10µl Spi-1 RT lysate + 100 fold excess competitor FP2 oligonucleotide; 5, 10µl Spi-1 RT lysate + 20 fold molar excess FP2 oligonucleotide; 6, 10µl Spi-1 RT lysate +100 fold molar excess of FP1 oligonucleotide. SI and SII refer to Spi-1 specific complexes. The lower mobility complexes are also present in the mock translation. Free probe can be seen at the bottom of the gel.

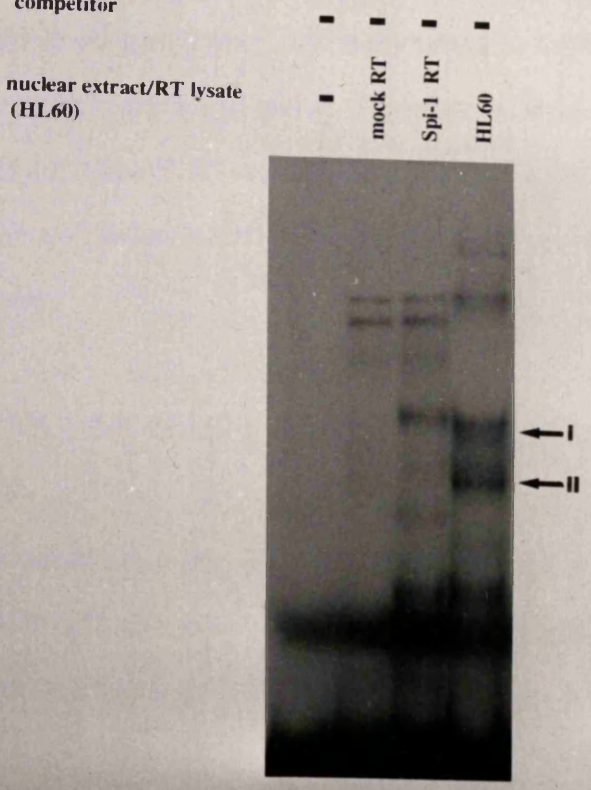
B: EMSA comparing the mobility of HL60 nuclear extract FP2 binding activities and Spi-1

Comparison of the mobility of the DNA binding activities present in HL60 nuclear protein to *in vitro* transcribed/translated Spi-1 by EMSA using FP2 radiolabelled probe. Lane 1, probe alone; 2, 10µl mock RT lysate; 3, 10µl Spi-1 RT lysate; 4, 10µg HL60 nuclear extract. Complexes generated by HL60 nuclear extract are labelled I and II. Free probe can be seen at the bottom of the gel.

A


competitor
nuclear extract/RT lysate (HL60)
-
-
-
-
mock RT
Spi-1 RT
Spi-1 FP2 ++++
Spi-1 FP2 +
Spi-1 FP1 ++++
SI
SII

B


competitor
nuclear extract/RT lysate (HL60)
-
-
-
-
mock RT
Spi-1 RT
HL60
I
II

8.5 Investigation of the FP3 DNA binding activities

The region protected by HL60 nuclear extract spanned -70 to -46 (figure 8.2) and was termed FP3. As with FP1 and FP2, the EMSA was used as an approach to investigate the DNA binding properties of these activities.

8.5 (i) Tissue distribution of the FP3 DNA binding activities

The tissue distribution of the FP3 DNA binding activity was assessed by EMSA using radiolabelled FP3 probe (figure 8.12). Using HL60 nuclear extract, two intense retarded complexes of low mobility are generated, a third low mobility complex is also sometimes generated but fails to produce consistent results in competition experiments. Complexes of similar mobilities are also generated with nuclear extract derived from U937, K562, Raji, and HeLa cell lines. Interestingly, similar activities are not generated using extracts derived from a neutrophilia and a CML patient's peripheral blood leukocytes; this is discussed in section 8.5 (ii). In addition to the factors generate complexes I and II, a number of other complexes are also generated, although not when HL60 nuclear extract is employed (figure 8.12). This suggests that a number of factors which are capable of binding this sequence *in vitro* are relatively common.

8.5 (ii) Changes in FP3 DNA binding activity during differentiation

An EMSA was carried out using nuclear extracts derived at different time points during induced HL60 differentiation using labelled FP3 as a probe. Considering the DMSO induction first (see figure 8.13 B), the three low mobility complexes I, II

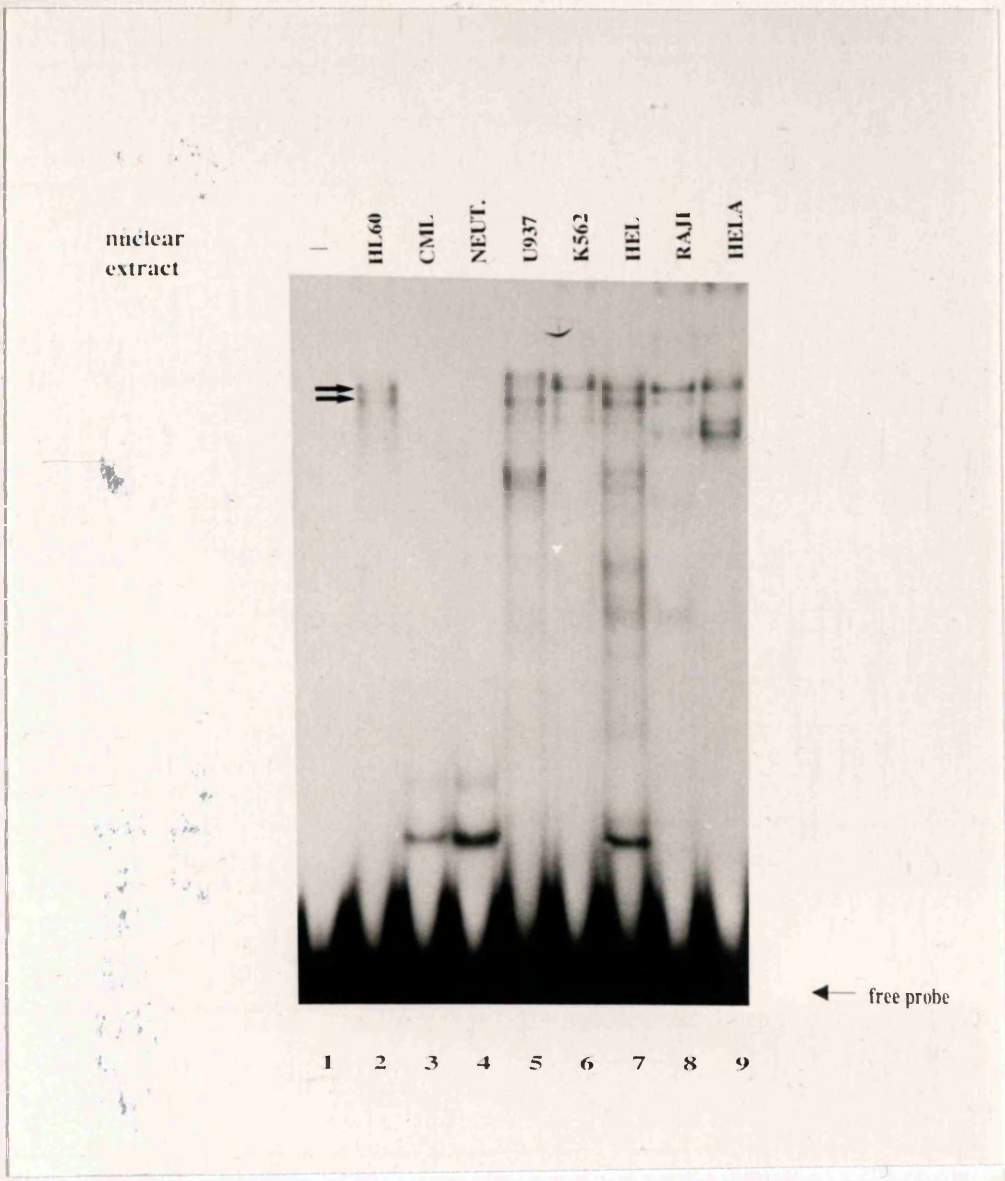


Figure 8.12 : EMSA of FP3 radiolabelled probe using nuclear extracts from a range of cell lines and patient leukocyte samples.

With the exception of lane **1**, all other lanes contain 10μg of nuclear extract from the following source: **2**, HL60; **3**, CML leukocytes; **4**, neutrophils derived from a neutrophilia patient; **5**, U937; **6**, K562; **7**, HEL; **8**, Raji; **9**, Hela. The arrows indicate the retarded complexes discussed in the text. Free probe can be seen at the bottom of the gel.

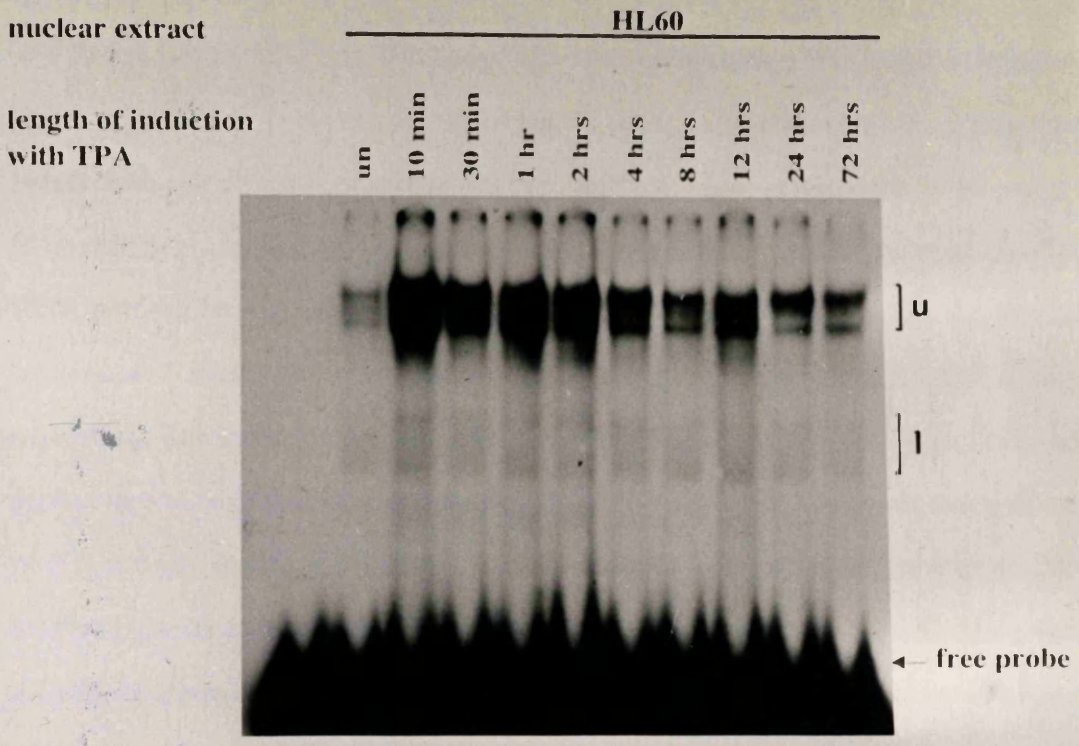
Figure 8.13 : EMSA showing changes in the FP3 binding activity during HL60 differentiation.

HL60 cultures were induced to differentiate using DMSO and TPA and nuclear protein was harvested at a range of time points as described in section 8.3(ii). These samples were used in the EMSA illustrated in this figure. With the exception of the left hand lane, each reaction contained 10μg of nuclear protein. un=uninduced HL60 nuclear extract, each of the other labelled lanes refers to the length of time post induction that the nuclear extract was harvested. u=upper complexes, l=lower complexes. Free probe can be seen at the bottom of the gel.

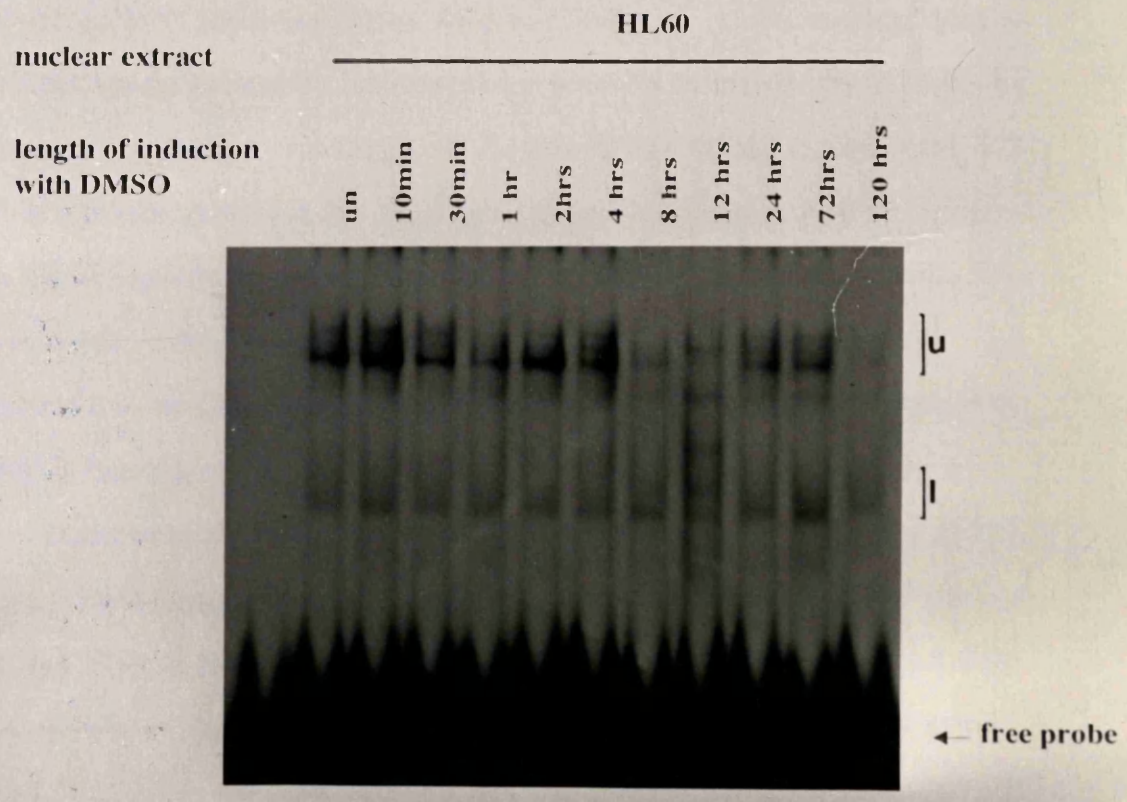
A: TPA induction.

B: DMSO induction.

A


nuclear extract
HL60
length of induction with TPA
un
10 min
30 min
1 hr
2 hrs
4 hrs
8 hrs
12 hrs
24 hrs
72 hrs
u
l
free probe

B


nuclear extract
HL60
length of induction with DMSO
un
10min
30min
1 hr
2hrs
4 hrs
8 hrs
12 hrs
24 hrs
72hrs
120 hrs
u
l
free probe

and III are labelled U, and all change proportionally during HL60 differentiation. There is little change in the activity of any of these complexes until the 5 day time point, with any differences before this point attributable to differences in the quality of the extracts, as this activity seemed particularly sensitive to degradation. As this DNA binding activity is particularly sensitive, it is pertinent to ask if the loss of activity seen after 3 days is a real effect or the consequence of degradation during preparation. The lack of any comparable FP3 binding activity in the extract derived from leukocytes from a neutrophilia patient (90% neutrophils) suggests that the loss of this activity in fully differentiated HL60 cells is a reflection of the lack of FP3 binding proteins as consequence of differentiation to a neutrophil-like cell. However, it could be argued that the fully differentiated cells contain relatively more proteases, which increases the degradation of this sensitive activity, during preparation of the nuclear extract.

If the FP3 binding activity is present until late in granulocytic differentiation, then the lack of FP3 binding activity in the CML sample is surprising. This sample, however, contained only 30% myelocytes with the majority of the remaining cells comprising PMNs and lymphocytes. As the FP3 binding activity is not present in fully differentiated neutrophils this would be expected to reduce the signal, although it should still be easily detectable. It may be that the ease of degradation of the FP3 binding activities resulted in loss of this activity in this sample as the CML samples are left at room temperature for some hours before they are obtained for analysis. The higher mobility complex labelled l remains relatively unchanged in intensity during differentiation, and this complex is present at the 5 day time point although slightly reduced in intensity.

Consideration of the TPA induction reveals a large rapid induction of FP3 binding activity (complexes labelled U) 10 minutes after treatment (see figure 8.13 A) of at least 14-fold. However as the most intense complex had clearly exceeded the linear response of the autoradiograph, this figure is an under estimation. The level of FP3 binding activity then falls back to levels 3-fold higher than in the uninduced cell

on the completion of differentiation. This large rapid increase of activity only seen when inducted to differentiate along the monocytic lineage is intriguing, but whether this activity plays a causal role in the process of differentiation remains to be seen. The higher mobility complexes L are transiently increased but then remain relatively unchanged.

8.5 (iii) Characterisation of the FP3 DNA binding

Examination of the FP3 sequence identified similarity to an Ets-binding site (see figure 8.10 B), which is remarkably similar to the *Drosophila* Ets-binding E74 sequence (Urness and Thummel 1990). A number of Ets binding sequences and a range of other oligonucleotides were tested to see if they could compete out the FP3 binding complexes in an EMSA. The result can be seen in figure 8.14 A. Considering complexes I and II, the oligonucleotide E74 was the most effective competitor. In addition, weak competition was observed using the oligonuceotides FP1, FP2, PU.I and PEA3, all of which are similar to Ets binding sites. Considering the relative affinities of these oligonucleotides for FP3 DNA binding activities I and II, the properties are similar to those of Ets-1, as the sequences which compete most efficiently are those which can bind Ets-1 with high affinity. Although less information is available concerning relative affinities of Ets-2 for different sequences, the information that is available suggests that it is similar to that of Ets-1 (Wasylyk *et al.*, 1990; Fisher *et al.*, 1991). Given the broader tissue distribution of Ets-2 (Bhat *et al.*, 1987) this is perhaps a stronger candidate for the FP3 binding complexes I and II than Ets-1.

Complex III gives inconsistent results on repeats for unknown reasons. Complex IV, has a similar mobility to FP2 binding complexes I and II, and its binding properties are similar to those of FP2 complex I (upper), which suggests they may be generated by the same transcription factors.

Figure 8.14 A: Analysis of the DNA binding properties of the FP3 binding activities using EMSA with a radiolabelled probe.

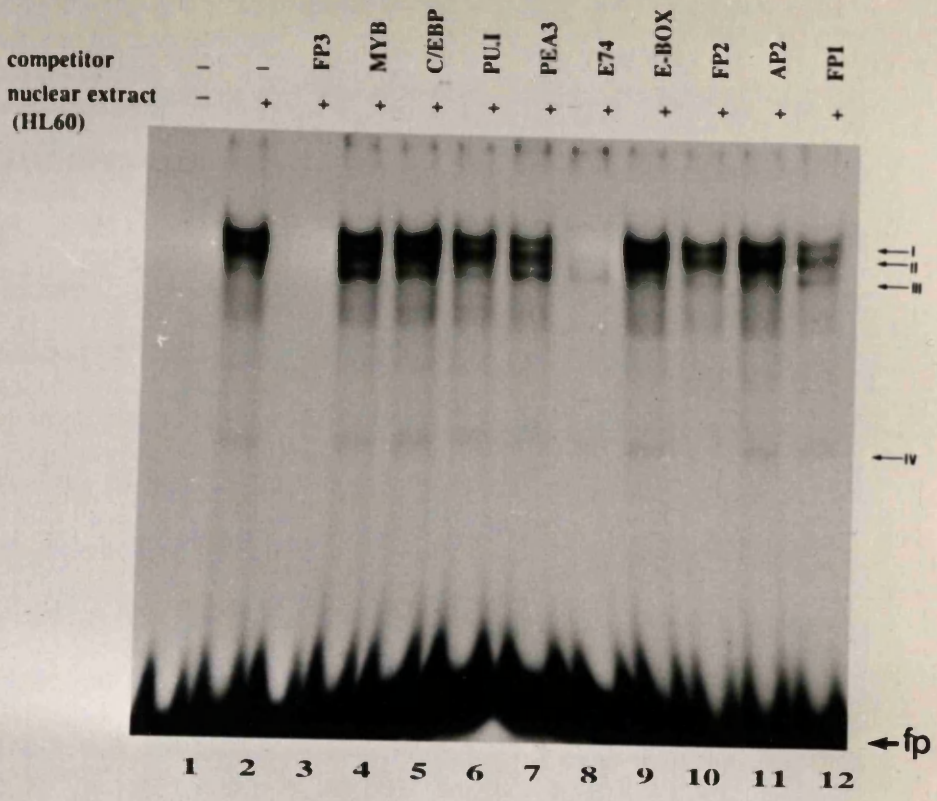
Each reaction with the exception of lane 1, contained 10μg of HL60 nuclear protein. Unlabelled competitor oligonucleotides were included (as indicated in the figure) at 100 fold molar excess. Retarded complexes are labelled, I, II, III and IV. Free probe can be seen at the bottom of the gel.
The sequence of the sense strand of the competitor oligonucleotides are listed

FP1: GGGTTCCCACCAAATTTCTCAACTGTCCTGCCGG
FP2: GGTAGATGAGAGGTTCCTCTGTGGAGTTCTACTTTAA
FP3: ACAGAAAGTAACCCCGGAAATTAGGACACCTCATCCC
AP2: CCGGCCCCAGGCGT (Oka *et al.*, 1991)
MYB: TTCGGCATAACGGTTCCGTAGCC (Watson *et al.*, 1992)
C/EBP: GCTGCAGATTGCGCAATCTGCAGC (Grove and Plumb 1994)
PU.1: CGTCCCAAGAGAGGAACCAATCAGCATTG (Klemz *et al.*, 1990)
E74: AGCTTCTCTAGCTGAATAACCCGGAAAGTAACTCATCGTCGA (Urness and Thummel 1990)
PEA3: GATCGAGGAAGTGAGTGAGTAACG (Martin *et al.*, 1988)

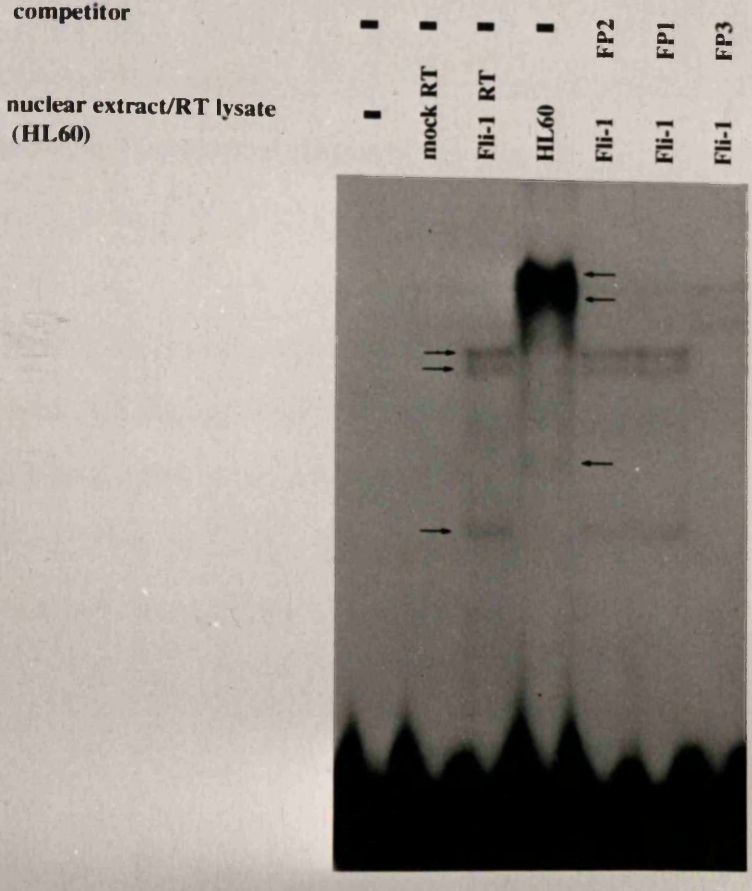
Figure 8.14 B: Fli-1 binds the FP3 sequence *in vitro*.

EMSA with FP3 radiolabelled probe comparing the complexes generated using Fli-1 (Nibbs *et al.*, 1994) primed reticulocyte lysate (RT) and HL60 nuclear extract. With the exception of lane 1, each lane contained either 10μl of RT lysate or 10μg of HL60 nuclear extract. Competitor oligonucleotides when included were at 100-fold molar excess as indicated in the figure. Lane 2, 10μl mock RT lysate; lane 3, 10μl Fli-1 RT lysate; lane 4, 10μg of HL60 nuclear extract; lane 5, 10μl of Fli-1 RT + FP2 competitor; lane 6; 10μl Fli-1 RT + of FP1 competitor and lane 7; 10μl of Fli-1 RT + FP3 competitor.

A


competitor
nuclear extract
(HL60)
– – FP3 MYB C/EBP PU.1 PEA3 E74 E-BOX FP2 AP2 FP1
– + + + + + + + + + + +
fp
1 2 3 4 5 6 7 8 9 10 11 12

B


competitor
nuclear extract/RT lysate
(HL60)
– – – – FP2 FP1 FP3
– mock RT Fli-1 RT HL60 Fli-1 Fli-1 Fli-1

8.5 (iv) Fli-1 binds the FP3 oligonucleotide

Another possible candidate for the FP3 DNA binding activities was the ErgB/Fli-1 (human/mouse) transcription factor, as this factor is expressed in HL60 cells (Watson *et al.*, 1992) and it binds to the E74 sequence with high affinity (Zhang *et al.*, 1993). In addition, although FP3 binding activities of similar mobility are present in a number of cell lines, the transfection data (see Chapter 9) suggest that this sequence may confer myeloid specificity and the *ergB* gene is expressed in a tissue-restricted fashion (Watson *et al.*, 1992). To test if this factor could bind the FP3 sequence, this protein was generated by *in vitro* transcription and translation in a reticulocyte lysate. A duplicate translation was carried out simultaneously containing ^{35}S-labelled methionine. An aliquot of this translation was fractionated on SDS-PAGE (4.21 ii) and autoradiography revealed that the majority of the label was incorporated into a doublet of equal intensity of apparent molecular weight around 50 kDa, which has been reported previously (Zhang *et al.*, 1993).

Referring to figure 8.14 B, it can be seen that the *fli*-1 programmed lysate can bind to the FP3 sequence and generate two low mobility complexes and a high mobility complex, which is a superficially similar pattern of complexes to that generated using HL60 nuclear extract, i.e. a low mobility doublet and a higher mobility complex. However, direct comparison to the complexes generated using HL60 nuclear extract, reveal that, although the pattern is the same, the Fli-1 complexes have a significantly higher mobility. It seems unlikely that the differences in mobility are the result of using the mouse *fli-1* cDNA as the template, as these proteins are 96% identical at the amino acid level, and most changes are conservative with predicted molecular weights of 51 kDa and 50.98 kDa for mouse and human respectively. However, the proteins may be modified post-translationally differentially in cells and cell free extracts which may affect their mobilities.

8.6 Ets-2 and PU.1 are expressed in this HL60 cell line.

As the strongest candidates for the FP2 and FP3 DNA binding activities are PU.1 and Ets-2, respectively, it was necessary to demonstrate that these factors are expressed in this particular cell line. In addition the other cell lines, HeLa, Raji and the T-cell line CEM were also included on this blot. This would allow the expression of the gene to be correlated with the results of both the DNA binding studies, and the activities of these sequences in transfection studies in these cell lines (Chaptet 9)

Figure 8.15 A reveals that *ets*-2 is expressed most highly in the HeLa cell line but is also expressed in the HL60 cells. Three transcripts can be detected, these are of sizes 3.5, 2.9 and 1.6 kb. A previous study of *ets*-2 expression in the HeLa cell line reported transcripts of 4.7, 3.2 and 2.7 kb present (Watson *et al.*, 1988). The reasons for the differences between the studies is unkown, although the HeLa cell line used in this study was HeLaS3 which is a subline which can grow in suspension.

In figure 8.15 B, the same filter was re-probed with Spi-1 (96% identical to the human PU.1 gene), reveals that this a single band of the expected size (Smith *et al.*, 1989) is present in the HL60 and the Raji cell lies. This result is as expected as this factor has a limited tissue distrubution and has been termed a macrophage and B-cell specific factor. The relatively low intensity of the band generated with the Raji RNA sample is partially the result of the comparative underloading of this sample compared to that of the HL60 sample.

Figure 8.15 C shows that the RNA was intact as this panel shows the result of re-probing the filter with a β-$_2$microglobulin probe as a loading control.

Figure 8.15: ets-2 and PU.1 are expressed in the HL60 cell line.

Each panel represent the same filter probed with

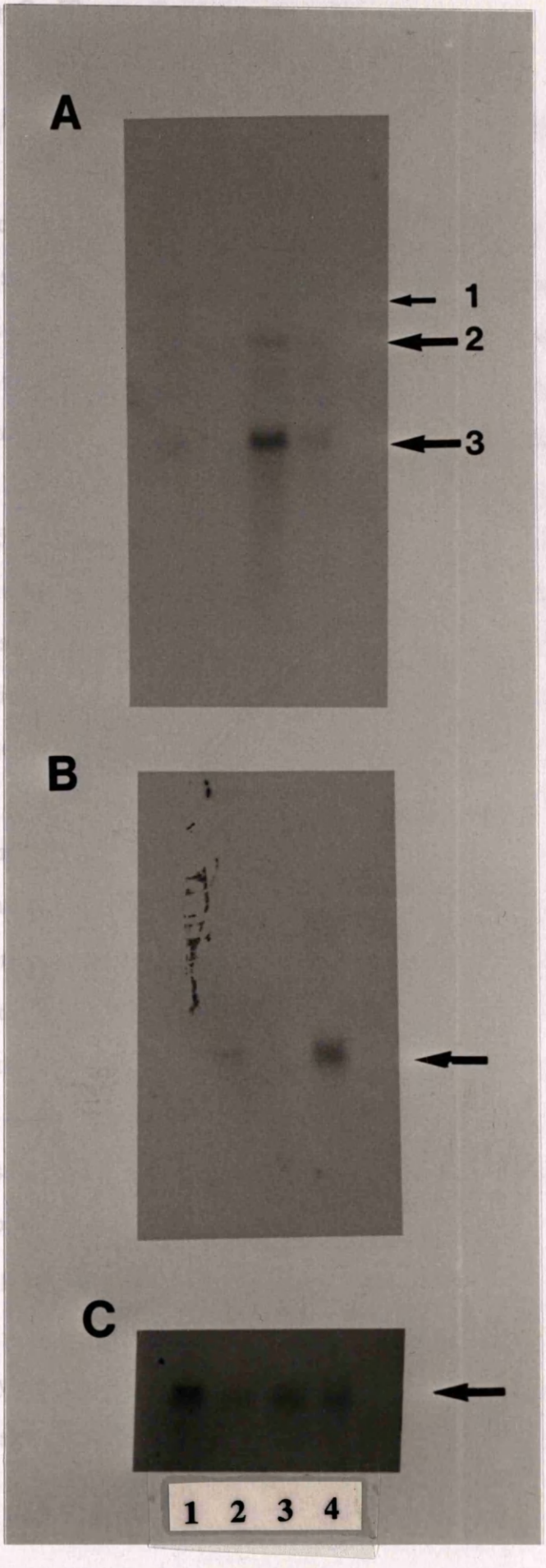
A: a full length *ets*-2 cDNA (Watson *et al.*, 1988)

B: a 400bp 5' PU.1 cDNA fragment (Smith *et al.*, 1991)

C: a full length β_2-microglobulin cDNA (Sugg *et al.*, 1981)

Each lane contains 15μg of RNA extracted from the following cell lines (from left to right) CEM, Raji, HeLa and HL60 (lanes 1-4).

Figure 8.15: ets-2 and PU.1 are expressed in HL60 cells



Chapter 9: Transfection studies

9.1 Optimisation of the conditions for transient transfection of HL60 cells

A necessary aspect of the work was the transfection of a myeloid cell line to test the transcriptional properties of a series of promoter deletion constructs. This was problematic as although a number of reports of transfection of the HL60 cell line have appeared in the literature, reproducibility between laboratories has been a problem and previous attempts in this group had proved unsuccessful. As these experiments were essential to link *in vitro* binding activities to transcriptional activities, an attempt to transfect and optimise the conditions for the HL60 cell line was undertaken.

For the initial experiments the reporter gene luciferase was used for two reasons: firstly, it has been reported to be the most sensitive of the available reporter genes (Williams *et al.*, 1989; Brasier *et al.*, 1989) and, secondly, this reporter gene has been used successfully in one report of transfection into HL60 cells and more extensively in the promonocytic cell line U937 (Pahl *et al.*, 1991; Pahl *et al.*, 1993; Yamada *et al.*, 1988). This gene was under the control of the SV40 promoter, which is an extremely powerful promoter and has been demonstrated to be active in HL60 cells (Reisman and Rotter 1989). The transfection protocol followed was electroporation using the conditions suggested by Dr Zhu Jing-de (personal communication) who was successfully transfecting the myelomonocytic mouse cell line WEHI, and the conditions employed are described in the section 4.20. The cells were harvested 18 hours post-transfection and a luciferase assay was carried out however, no luciferase activity could be detected in these cells.

One possible explanation as to why HL60 cells are so difficult to transfect is that they are phagocytic cells whose function is to ingest and degrade foreign particles, consequently the cytoplasm is full of degradative enzymes. As the protocol for the assay of most reporter genes involves the lysis of the cells as a first step, this will

release proteases from intracellular granules. It had been noted that the generation of intact nuclear protein from myeloid cell lines and HL60 in particular was difficult. This problem with proteolytic cleavage had been previously noted by other workers (Galson and Housmann 1989). To overcome this problem, a secreted reporter gene rather than luciferase was used. Secreted reporter genes had been used successfully (M.Grove personal communication) in transfecting the mouse macrophage RAW cell line at high efficiency. These cells also have abundant proteases stored in the cytoplasm. The secreted placental alkaline phosphatase gene was employed as a reporter gene (Henthorn *et al.*, 1988), as the assay does not involve lysis and release of cytoplasmic proteases. A repeat of the previous experiment was undertaken using the alkaline phosphatase reporter gene cotransfected with the luciferase reporter gene. The protocol was as described in the previous experiment, with 10μg of pSV2ASPAP and 10μg of pGL2-promoter plasmids. In addition, the cell line HeLaS3 was also transfected as a positive control for the luciferase assay. Given that the lysis of the cells may generate proteolysis, the luciferase assay was carried out using a cocktail of protease inhibitors (section 4.16 (i). Although no luciferase activity could be detected, alkaline phosphatase activity was easily detected in the medium (data not shown), suggesting that either the alkaline phosphatase assay is more sensitive than the luciferase assay or that degradation of the luciferase protein on lysis of the cells is a problem.

Comparison of the results of the HeLa and HL60 cell lines revealed that the efficiency of transfection into HL60 was significantly lower than that into the HeLa cell line. However, given a successful (if low efficiency protocol), it was possible to optimise the conditions for this cell line. Initially, the voltage employed was varied between 125V and 350V however no improvement on the 250V originally used could be detected. Similarly, the capacitance of 960μF could not be improved upon. Increasing the amount of DNA however, significantly increased the transfection efficiency in a linear fashion between 5 and 20μg (figure 9.1).

The alkaline phosphatase activity is very stable, and the level of this activity should accumulate in the medium with time, implying that the longer post transfection

Figure 9.1 A: The effect of plasmid concentration on efficiency of transfection into HL60 cells. Electroporation at 250V, 960μF of $5x10^6$ HL60 cells was carried out as described in secion 4.20. The reporter plasmid pSVA2SPAP was included at concentrations of 0, 5,10 and 20ug. The medium was assayed for heat stable alkaline phoshatase activity 18 hours post transfection. The assay was allowed to develop for 2 hours, and then the OD at 495nm was measured. The average activity from triplicate samples in two independent experiments is represented in the histogram.

Figure 9.1 B: The effect of carrier DNA on the efficiency of transfection into HL60 cells. Electroporation was carried out as decribed in figure 9.1A. Transfections were carried out using 45μg of bluescript (BS); 5μg of pSVA2SPAP plasmid (AP); 5μg of pSVA2SPAP and 45μg of bluescript (AP+BS); 5μg of pSVA2SPAP and 45μg of sonicated salmon sperm DNA (AP+ssDNA). The phosphatase assay was allowed to develop for 2 hours and then the OD at 495nm was measured. The average activity of triplicate samples from two independent experiments are represented in the histogram.

Figure 9.1 A: The effect of plasmid concentration on the transfection efficiency of HL60 cells

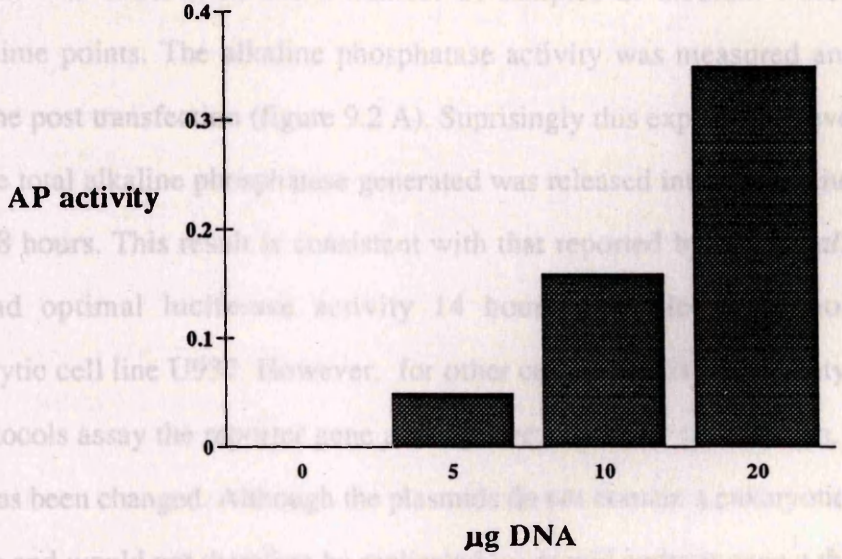
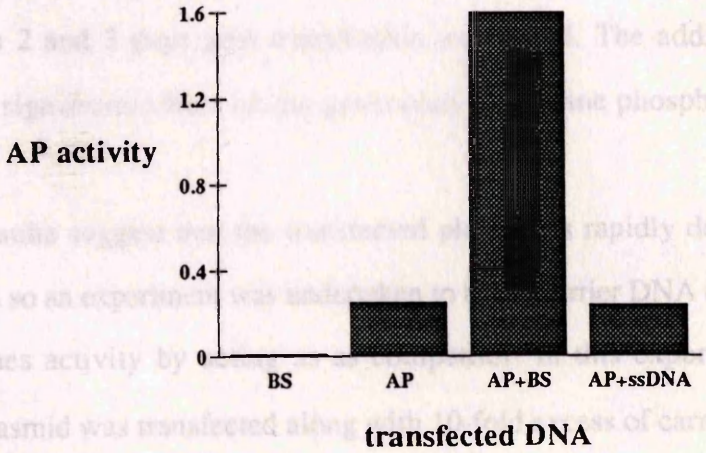


Figure 9.1 B: The effect of carrier DNA on the efficiency of transfection into HL60 cells



the medium is tested the greater the activity. It important to determine the optimal time point to sample the medium after the transfection, as it was necessary to sample the medium when the highest activity could be obtained but prior to the loss of a linear increase in activity. To determine a time course of alkaline phosphatase activity, a transfection was undertaken and a number of samples of medium were taken at different time points. The alkaline phosphatase activity was measured and plotted against time post transfection (figure 9.2 A). Suprisingly this experiment revealed that 70% of the total alkaline phosphatase generated was released into the medium within the first 18 hours. This result is consistent with that reported by Pahl *et al.*, (1991), who found optimal luciferase activity 14 hours post electroporation of the promonocytic cell line U937. However, for other cell lines this result is atypical, as many protocols assay the reporter gene activity five days after transfection, after the medium has been changed. Although the plasmids do not contain a eukaryotic origin of replication and would not therefore be replicated we would perhaps expect the plasmid to remain transcriptionally active for a longer period. Clearly the rapid loss of reporter gene activity could be explained by rapid loss of the plasmid by degradation. An alternative explanation is that the SV40 promoter shows a serum response in this cell line, as the transfection procedure involves the addition of some fresh medium (1ml of fresh medium to 4ml of conditioned medium). To eliminate this possibility, the effect of the addition of replacement of the medium with fresh or conditioned medium to transfected cells 2 and 3 days post transfection was tested. The addition of fresh medium had no significant effect on the generation of alkaline phosphatase activity (figure 9.2 B).

These results suggest that the transfected plasmid is rapidly destroyed after entering the cells so an experiment was undertaken to test if carrier DNA could increase the reporter genes activity by acting as as competitor. In this experiment 5µg of pSV2ASPAP plasmid was transfected along with 10-fold excess of carrier DNA. The result can be seen in figure 9.1 B, which clearly illustrates a large increase in activity with the inclusion of 10-fold excess of the bluescript plasmid, although the inclusion

Figure 9.2 A: Time course of the generation of alkaline phosphatase activity post transfection. HL60 cells were electroporated with 5µg of pSVA2SPAP and 45µg of bluescript as described in section 4.20. Samples of medium were taken from the transfected cells at the time points indicated in the figure. The alkaline phosphatase activity was measured and the results are presented as a proportion of maximum activity. The average activities of four and three duplicates from two independent transfections are represented in the histogram.

Figure 9.2 B: The effect of serum stimulation on the SV40 promoter in HL60 cells. HL60 cells were transfected with 5µg of pSVA2SPAP and 45µg of bluescript as described in section 4.20, with the exception that each transfectant was split into two flasks after electroporation. The samples were then given the following treatments. D2, these samples were resuspended in conditioned medium two days post transfection. D3, these samples were resuspended in conditioned medium three days post transfection. D2+medium, was resuspended in fresh medium two days post transfection. D3+medium, was resuspended in fresh medium 3 days post transfection. Samples of medium were taken 24 hours after this change in medium. The phosphatase assay was allowed to develop for 8 hrs and then the OD at 495nm was measured . The average activities of the results of triplicate tranfections from two independent experiments are illustrated in the histogram.

Figure 9.2A : Time course of alkaline phosphatase activity post transfection of HL60 cells.

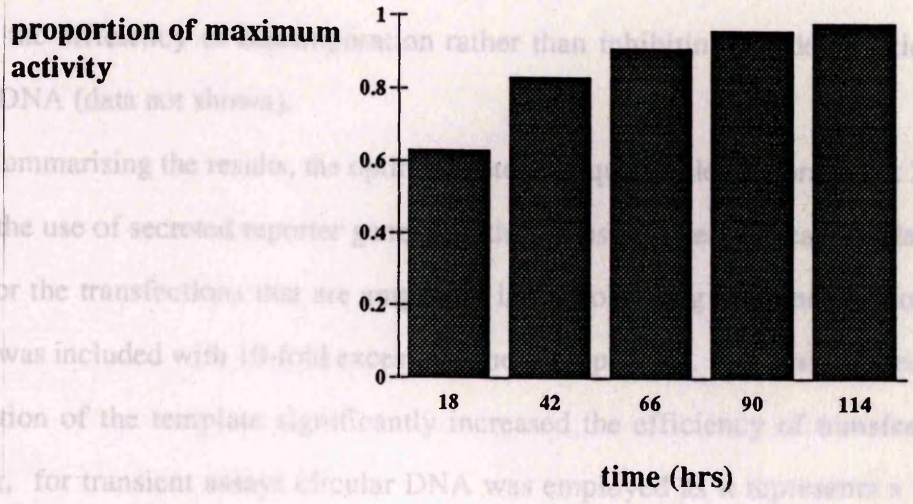
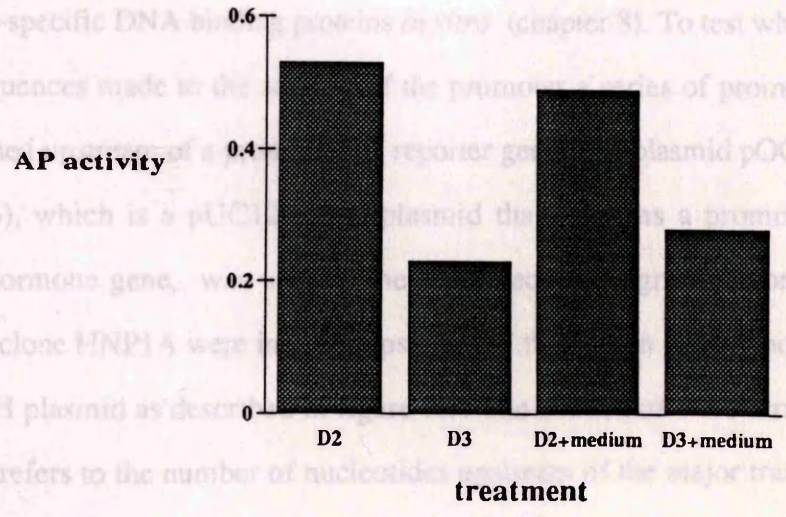


Figure 9.2 B : The effect of serum stimulation on the SV40 in HL60 cells.



of sonicated salmon sperm DNA had no effect. By what mechanism the inclusion of carrier plasmid DNA increases the alkaline phosphatase activity is unknown, although the the inclusion of excess plasmid had no effect on the time course of relative alkaline phosphatase activity generation, perhaps suggesting that the increase in DNA may increase the efficiency of electroporation rather than inhibiting the degradation of plasmid DNA (data not shown).

Summarising the results, the optimal protocol requires: electroporation at 250V 960uF, the use of secreted reporter genes and the inclusion of excess carrier plasmid DNA. For the transfections that are employed in the following sections, 5μg of test plasmid was included with 10-fold excess of bluescript plasmid. It was also noted that linearisation of the template significantly increased the efficiency of transfection. However, for transient assays circular DNA was employed as it represents a more physiological template. Although no rigorous experiments were undertaken to test the observation, it is clear that the cells must be growing optimally for high efficiency transfection into this cell line.

9.2 Generation of a series of reporter constructs

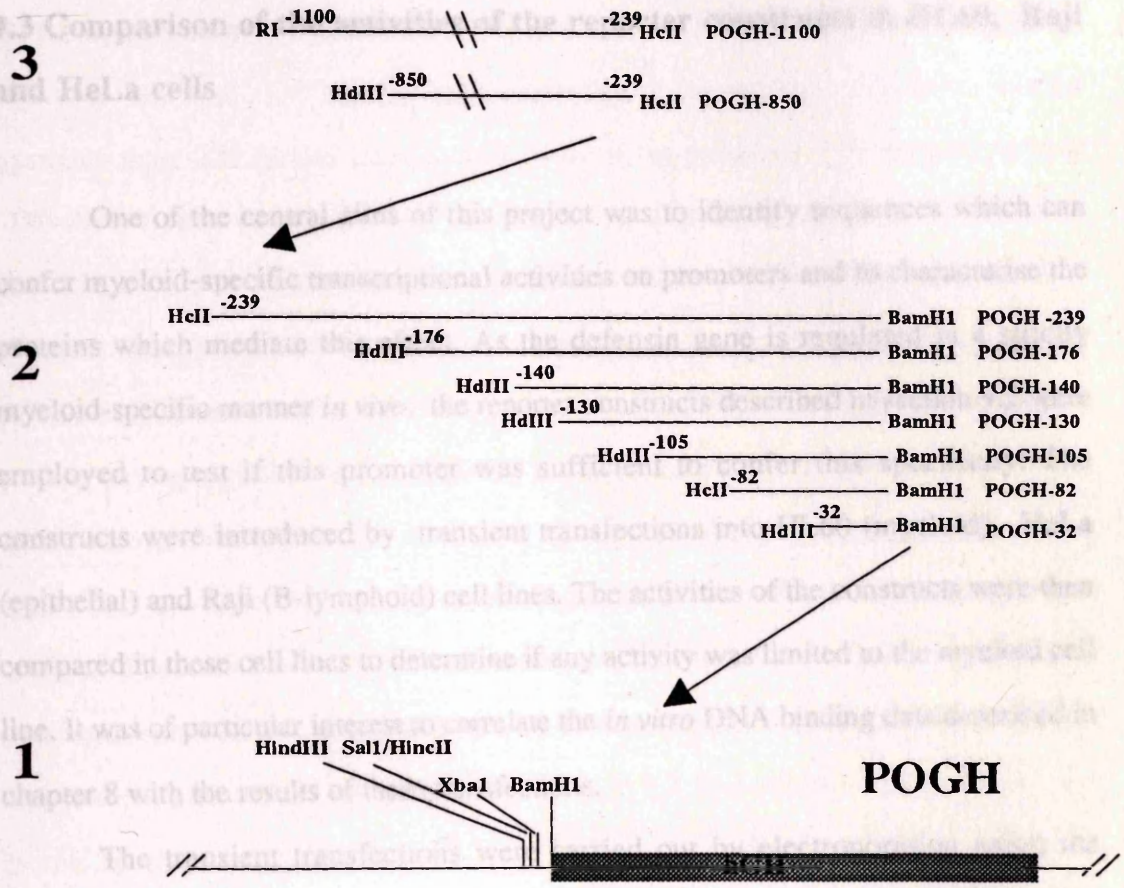
A number of sites in the defensin promoter were identified as interacting with sequence-specific DNA binding proteins *in vitro* (chapter 8). To test what contribution these sequences made to the activity of the promoter a series of promoter fragments were cloned upstream of a promoterless reporter gene. The plasmid pOGH (Snelden *et al.*, 1986), which is a pUC12 based plasmid that contains a promoterless human growth hormone gene, was used as the basic vector. Fragments from the defensin genomic clone HNP1A were inserted upstream of the human growth hormone gene in the pOGH plasmid as described in figure 9.3. The constructs were termed pOGH-X, where X refers to the number of nucleotides upstream of the major transcription start site of the defensin gene cloned into the pOGH vector. The constructs in figure 9.3 2 were generated by PCR from a plasmid template containing an HNP1A genomic clone

Figure 9.3: Schematic representation of the generation of the reporter constructs used in this study.

1 The promoterless pUC12 based pOGH plasmid containing the human growth hormone gene was used as a basic vector. Fragments of the defensin promoter were cloned in front of the human growth hormone gene using the restriction sites shown in the diagram. **2** Fragments overlapping the site of transcriptional initiation were generated by PCR using a plasmid template containing a defensin HNP1A genomic clone of the upstream regions. The primers contained restriction sites at the 5' ends and were used to clone these fragments (these oligonucleotides were 20bp in length, with the nucleotide position X representing the first of these 20 bp. Inaddition, each oligonucleotide also had a restriction site 5' to this sequence) into the pOGH vector. These constructs were labelled pOGH-X where X refers to the number of nucleotides upstream of the transcriptional start site cloned in front of the human growth hormone gene. **3** Two other constructs were generated using pOGH-239 as a vector. Fragments isolated by restriction digestion of a defensin HNP1A genomic clone were cloned into the HincII site of pOGH-239 after they had been blunt-ended by klenow filling.

RI = EcoRI
HdIII = HindIII
HcII = HincII

Figure 9.3: Schematic representation of the genration of reporter gene constructs used in this study.



using the oligonucleotides with restriction site ends. All fragments generated by PCR were sequenced on both strands after cloning. The constructs pOGH-1100 and pOGH-850 were generated by cloning inserts into the HincII site of pOGH-239. The inserts were derived by HincII/EcoRI or HincII/HindIII digestion of the HNP1A genomic clone. The fragments were blunt-ended by klenow filling in the restriction site ends prior to cloning into the pOGH-239 HincII digested vector.

9.3 Comparison of the activities of the reporter constructs in HL60, Raji and HeLa cells

One of the central aims of this project was to identify sequences which can confer myeloid-specific transcriptional activities on promoters and to characterise the proteins which mediate this effect. As the defensin gene is regulated in a strictly myeloid-specific manner *in vivo*, the reporter constructs described in section 9.2 were employed to test if this promoter was sufficient to confer this specificity. The constructs were introduced by transient transfections into HL60 (myeloid), HeLa (epithelial) and Raji (B-lymphoid) cell lines. The activities of the constructs were then compared in these cell lines to determine if any activity was limited to the myeloid cell line. It was of particular interest to correlate the *in vitro* DNA binding data described in chapter 8 with the results of these transfections.

The transient transfections were carried out by electroporation using the conditions described in section 9.1. In addition to the reporter constructs, 5µg of the pSV2ASPAP plasmid was added to allow variations in transfection efficiency to be corrected. To allow comparison of the activities of different constructs between cell lines, it is necessary to have a standard reference promoter against which they can be compared. By taking a promoter which has a similar level of activity in all cell types it is possible to compare the activity of different constructs in a number of cell lines by comparison of the activities of these constructs relative to this standard promoter. For the purpose of this study the herpes simplex virus thymidine kinase promoter was used

in the pTKGH plasmid. This promoter is very commonly used in studies of this nature, as it is assumed to have equal activity in the cell lines tested, but it is worth bearing in mind that it is unlikely that any promoter has the same level of activity in all cell types.

9.3 (i) HL60

The results of transfecting HL60 cells are illustrated in figure 9.4. Most notable is that the construct pOGH-82 has the highest activity, and the inclusion of sequences between -105 and -130 repress the reporter gene expression. Sequences further upstream than -239 further repress the activity of the promoter. The region spanning -130 to -140 may contain a weak positive-acting sequence, although its full effect could be obscured by the repression generated by the -105 to -130 sequence. The simplest interperation of these results is that the reason for the high activity of the pOGH-82 construct is a result of the inclusion of the FP3 sequence, which binds a positive-acting regulator of transcription. In addition the smallest construct pOGH-32, which includes the proximal TATA box, up-regulates the expression of the reporter gene. This could be due simply to the inclusion of a TATA box which acts as a minimal promoter. Interestingly, immediately 3' of the TATA box is a putative Ets-binding sequence, whose sequence suggests that it could bind PU.1. This sequence can be seen to be weakly footprinted in the figure 8.2, but whether this plays any role *in vivo* remains to be seen.

The low level of activity seen when the sequences between -130 and -105 are included in the reporter construct suggests that the FP1 element may act as a repressor element in this promoter. This would be an intriguing possibility given that the FP1 binding activity is rapidly down-regulated only on granulocytic differentiation, which corresponds to the expression of this gene. Inclusion of the 5' TATA box within the sequences -105 and -82 (see figure 8.2 B) also results in a reduced activity in comparison to that seen using the pOGH-82 fragment. It could be argued that the presence of this TATA box results in a change in the site of transcriptional initiation

Figure 9.4: Histogram illustrating the average activity (table 9.4) of the pOGH-defensin reporter constructs after transient transfection into HL60 cells. Each bar represents the average activity of duplicate transfections in 8 independent experiments. The cartoon indicates the positions of the footprints in relation to the promoter reporter constructs.

Table 9.4: Human growth hormone activity of the pOGH-defensin promoter constructs after transient transfection into HL60 cells. Xμg of the reporter construct (for pOGH, 5μg was added and for each of the other constructs an equivalent molar amount was added), 5μg of pSV2ASPAP and bluescript to a total of 50μg were cotranfected into HL60 cells as described in section 9.2. The medium was sampled 18 hours post transfection and 100μl was assayed for human growth hormone activity by radioimmunoassay as described in chapter 4.20(iii). The figures represent counts per minute after normalising transfection efficiency using the pSV2ASPAP as an internal standard. The results are from 8 independent experiments, and the average and standard deviation are indicated in the table.

Figure 9.4: Illustrating the average activity of the pOGH-defensin promoter constructs after transfection into HL60 cells

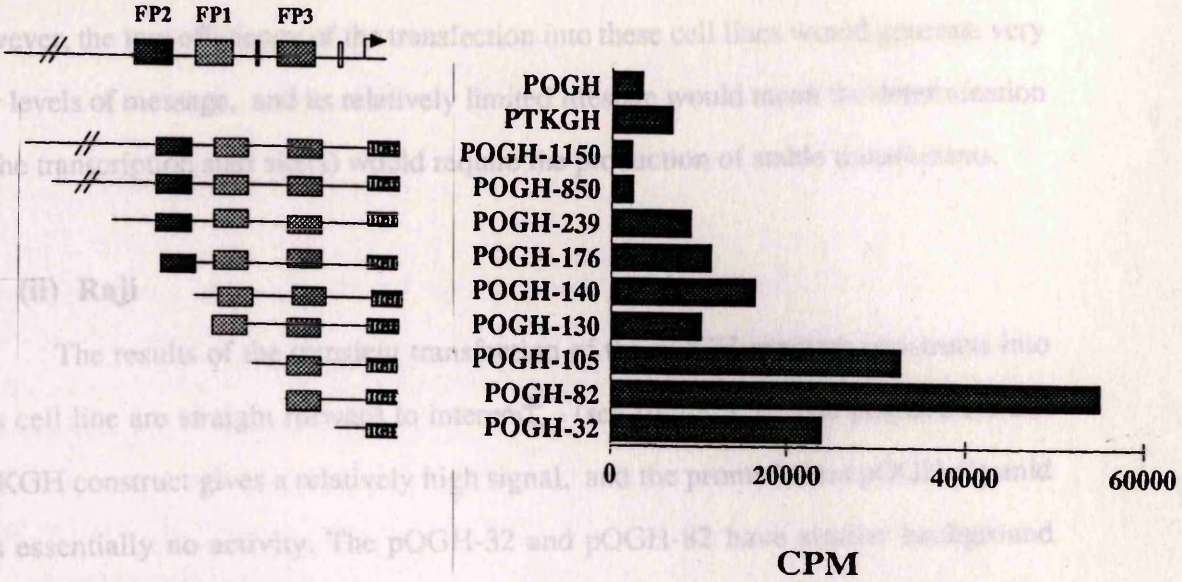


Table 9.4: Human growth hormone activity of HL60 cells transiently transfected with pOGH-defensin reporter constructs

	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP8	Averag	Std dev
POGH	5049	3176	4892	3060	2131	3221	4123	3076	3591	1006
PTKGH	6715	5122	9849	6157	5157	7664	8753	6994	7051	1661
POGH-1150	4127	3171	2111	1013	2117	2189	2953	2011	2461	936
POGH-850	3156	2233	1534	3080	1943	2790	3122	2315	2521	607
POGH-239	13725	10114	7894	4960	9768	8795	8893	8699	9106	2442
POGH-176	14132	11013	8943	11593	11042	10187	12376	11378	11333	1521
POGH-140	23992	21402	21707	14321	10143	11405	11578	16571	16389	5378
POGH-130	11978	9155	7768	15231	8543	7143	10687	12321	10353	2728
POGH-105	43171	40111	37442	23453	30922	27685	28754	31201	32842	6745
POGH-82	68921	63125	50143	40146	55894	52690	49885	61032	55229	9041
POGH-32	29460	32130	27485	11043	21031	23987	21087	24536	23844	6473

and the reduced level of transcription could be a result of interference by the attempted assembly of two preinitiation complexes. Clearly to determine if this is the case the site(s) of transcriptional initiation of these constructs needs to be determined; however, the low efficiency of the transfection into these cell lines would generate very low levels of message, and its relatively limited lifespan would mean the determination of the transcription start site(s) would require the production of stable transfectants.

9.3 (ii) Raji

The results of the transient transfection of the pOGH-reporter constructs into this cell line are straight forward to interpret (see figure 9.5). The positive control pTKGH construct gives a relatively high signal, and the promoterless pOGH plasmid has essentially no activity. The pOGH-32 and pOGH-82 have similar background levels of expression as the pOGH plasmid. All of the larger constructs have essentially no promoter activity, repressed below even that seen with the promoterless pOGH. These results suggest that this promoter has no activity in B-cells.

9.3 (iii) HeLa

The results of the transient transfection of the pOGH-defensin promoter constructs into this cell line can be seen in figure 9.6. The interpretation of the results generated using this cell line are complicated by the high level of activity of the pOGH construct in this cell line, which is similar to that seen in the HL60 cell line. However, although in the HL60 cell line the defensin promoter had significantly higher activity than the pOGH construct, this is not seen when the constructs are introduced into HeLa cells. As can be seen in the figure the inclusion of the sequence containing FP3 results in a small up-regulation of reporter gene activity. Inclusion of sequences further upstream reduce the activity of the reporter gene to below the level of the promoterless pOGH. As the activity of the constructs in this cell line are all lower than those with the tk promoter, the most straightforward explanation of the results is that the defensin promoter shows a much elevated level of expression in the myeloid (HL60) rather than

Figure 9.5: Histogram illustrating the activity (table 9.5) of the pOGH-defensin reporter constructs after transient transfection into Raji cells. Each bar represents the average of four independent experiments.

Table 9.5: Human growth hormone activity of the pOGH-defensin promoter constructs after transient transfection into Raji cells. Xμg of the reporter construct (for pOGH, 5μg was added and for each of the other constructs an equivalent molar amount was added), 5μg of pSV2ASPAP and bluescript to a total of 50μg were cotranfected into Raji cells by electroporation using the same conditions as those described for HL60 cells. The medium was sampled 18 hours post transfection and 100μl was assayed for human growth hormone activity by radioimmunoassay as described in chapter 4.20(iii). The figures represent counts per minute after normalising transfection efficiency using pSV2ASPAP as an internal standard. The results are from 4 independent experiments, and the average and standard deviation are indicated in the table.

Figure 9.5: Illustrating the average activity of the pOGH-defensin reporter constructs into Raji cells

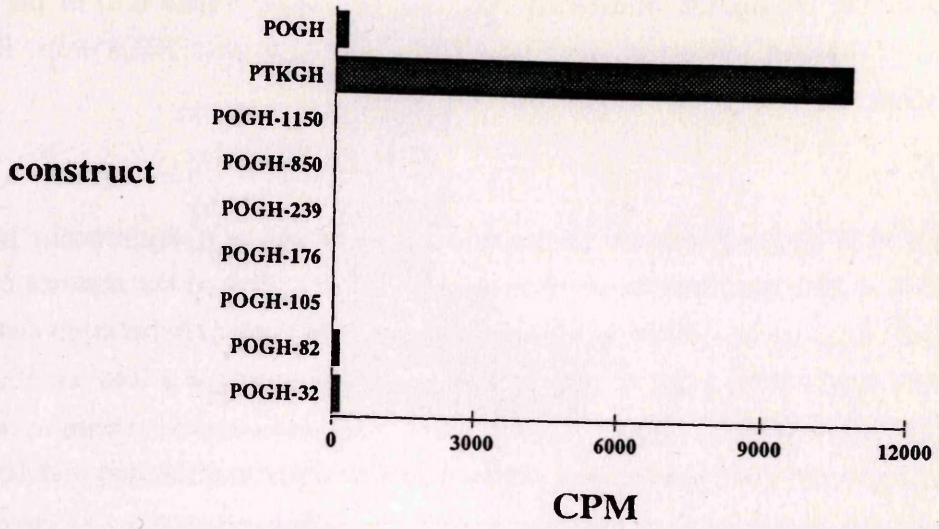


Table 9.5: Human growth hormone activity of Raji cells transiently transfected with the pOGH-defensin promoter constructs

	EXP1	EXP2	EXP3	EXP4	AVE	SD
POGH	313	400	60	317	272	147
PTKGH	13424	14005	3523	12432	10849	4926
1150	0	5	0	0	1	3
850	25	30	0	0	14	16
239	8	0	0	5	3	4
176	34	20	0	15	17	14
105	49	25	0	14	22	20
82	262	300	0	120	170	137
32	250	300	37	125	178	119

Figure 9.6: Histogram illustrating the average activity (table 9.6) of the pOGH-defensin reporter constructs after transient transfection into HeLa cells. Each bar represents the average of 7 independent experiments.

Table 9.6: Human growth hormone activity of the pOGH-defensin promoter constructs after transient transfection into Hela cells. Xμg of the reporter construct (for pOGH, 5μg was added and for each of the other constructs an equivalent molar amount was added), 5μg of pSV2ASPAP and bluescript to a total of 50μg were cotranfected into HeLa cells by electroporation using the same conditions as described for HL60 cells. The medium was sampled 18 hours post transfection and 100μl was assayed for human growth hormone activity by radioimmunoassay as described in chapter 4.20(iii). The figures represent counts per minute after normalising transfection efficiency using the pSV2ASPAP as an internal standard. The results are from 7 independent experiments, and the average and standard deviation are indicated in the table.

Figure 9.6: Illustrating the average activity of the pOGH-defensin promoter constructs after transient transfection into HeLa cells

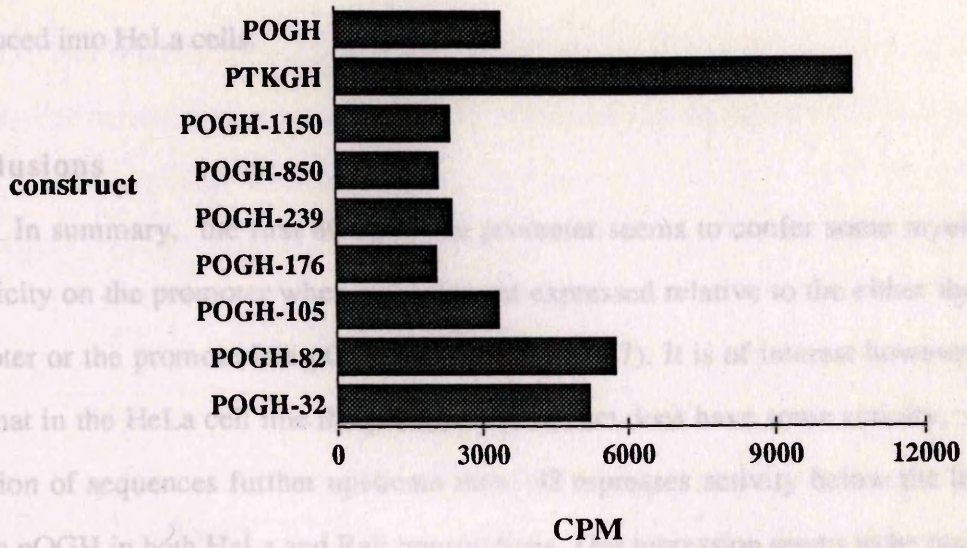


Table 9.6: Human growth hormone activity of HeLa cells transiently transfected with the pOGH-defensin reporter constructs

	exp1	exp2	exp3	exp4	exp5	exp6	exp7	averag	std de
POGH	4101	2330	3392	3632	3219	4122	3367	3451	609
PTKGH	12873	8976	11134	9810	10167	8975	12456	10627	1580
POGH-1150	2101	1899	2011	2132	2580	3101	2936	2394	478
POGH-850	1299	2123	1997	1856	3108	2310	2256	2135	545
POGH-239	2290	2134	2101	1865	3102	2749	2693	2419	439
POGH-176	2789	1228	1992	2256	1863	2184	2394	2100	486
POGH-105	4001	2430	3011	2662	3145	4389	3927	3366	742
POGH-82	7197	4511	5677	6227	6753	5219	4845	5775	997
POGH-32	5113	3122	4886	5112	6219	6391	5871	5244	1104

the epithelial (HeLa) compartment. A high level of pOGH activity is seen in both HeLa and HL60 cell lines, although in the latter cell line the defensin promoter has a much greater activity than pOGH, this is not seen when the reporter constructs are introduced into HeLa cells.

Conclusions

In summary, the first 80 bp of the promoter seems to confer some myeloid specificity on the promoter when activities are expressed relative to the either the tk promoter or the promoterless pOGH plasmid (table 9.7). It is of interest however to note that in the HeLa cell line the pOGH-82 construct does have some activity, and inclusion of sequences further upstream than -82 represses activity below the level seen in pOGH in both HeLa and Raji transfections. This repression seems to be mainly a result of the inclusion of the sequences -82 to -105, which include the 5' TATA box. It could be that the attempt to assemble two preinitiation complexes results in the interference of the low level of transcription generated in these cell lines using the small constructs.

Two of the sequences which had been indentified as footprints using HL60 nuclear extract have significant effects on the expression of the reporter gene. The construct pOGH-82 includes the FP3 sequence and shows the highest activity in HL60 cells, suggesting that the FP3 binding activities are positive regulators of this sequence. The inclusion of the FP1 sequence in the construct pOGH-130 leads to a significant reduction in the activity of this promoter suggesting that the FP1 binding activities are acting as a repressor. The effect of the FP2 sequences may well be masked by the repressive effect of the FP1 sequence which significantly reduces expression. The other sequences which were of interest were the putative c-Myb binding sites, although c-Myb binding activity could not be detected in nuclear extracts. Further experiments were undertaken to investigate the role of c-Myb in the regulation of this gene.

Table 9.7: The relative activities of the pOGH-defensin promoter constructs in the cell lines. The activities are expressed relative to the activity of the pTKGH plasmid, which is given an arbitrary value of 1 in each cell line.

	HL60	Raji	Hela
PTKGH	1.00	1.000	1.00
POGH-1150	0.35	0.000	0.23
POGH-850	0.36	0.001	0.20
POGH-239	1.29	0.000	0.23
POGH-176	1.60	0.002	0.20
POGH-140	2.32	-	-
POGH-130	1.47	-	-
POGH-105	4.65	0.002	0.32
POGH-82	7.80	0.016	0.54
POGH-32	3.34	0.016	0.49
POGH	0.51	0.025	0.37

9.4 (i) The defensin gene is a target for c-Myb transactivation

Given that the promoter contained two potential c-Myb binding sites (figure 8.1), and its expression pattern suggests that this gene would be a good candidate gene for regulation by c-Myb, it was decided to test reporter constructs to determine if they could be transactivated by exogenous c-Myb. The expression construct pJ4myb containing a full length mouse cDNA under the control of the Moloney murine leukaemia virus LTR , and a similar construct with a small deletion which prevents c-Myb from binding to DNA (pJ4mybΔR3), were kindly provided by Dr.R.Watson. In addition the Myb binding sites in the pSVnMCAT plasmid (provided by Dr.R.Watson) were removed by restriction enzyme digestion and cloned upstream of the tk promoter to act as a positive control for c-Myb responsiveness.

A series of the pOGH-defensin promoter constructs described in section 9.2 were co-transfected with the c-Myb expression vector (5μg of pOGH-X; 5μg of expression vector; 5μg of pSV2ASPAP) into the HL60 cell line. This cell line already contains endogenous c-Myb, but the inclusion of the sequence between -130 and -105 which contained the putative c-Myb binding site acted as a repressor in the transient transfection experiments. A hypothesis to explain these results is that the C/EBP-like factor that binds this sequence *in vitro* is a repressor which is rapidly lost on differentiation, allowing c-Myb to bind and activate expression of the gene. By elevating the levels of c-Myb by the introduction of an expression vector we might expect to relieve this repression. The results of this experiment can be seen in figure 9.8. Clearly the expression of exogenous c-Myb in this cell line transactivates the reporter constructs which contain a single c-Myb binding site (pOGH-130, -176 and -239). The transactivation is of the order of around 3.5-fold. In addition, the largest construct tested (pOGH-850) contains a second potential c-Myb binding site centered around nucleotide -240, and is up-regulated approximately 5-fold by exogenous c-

Figure 9.7: FP1 - a composite C/EBP-Myb binding sequence?

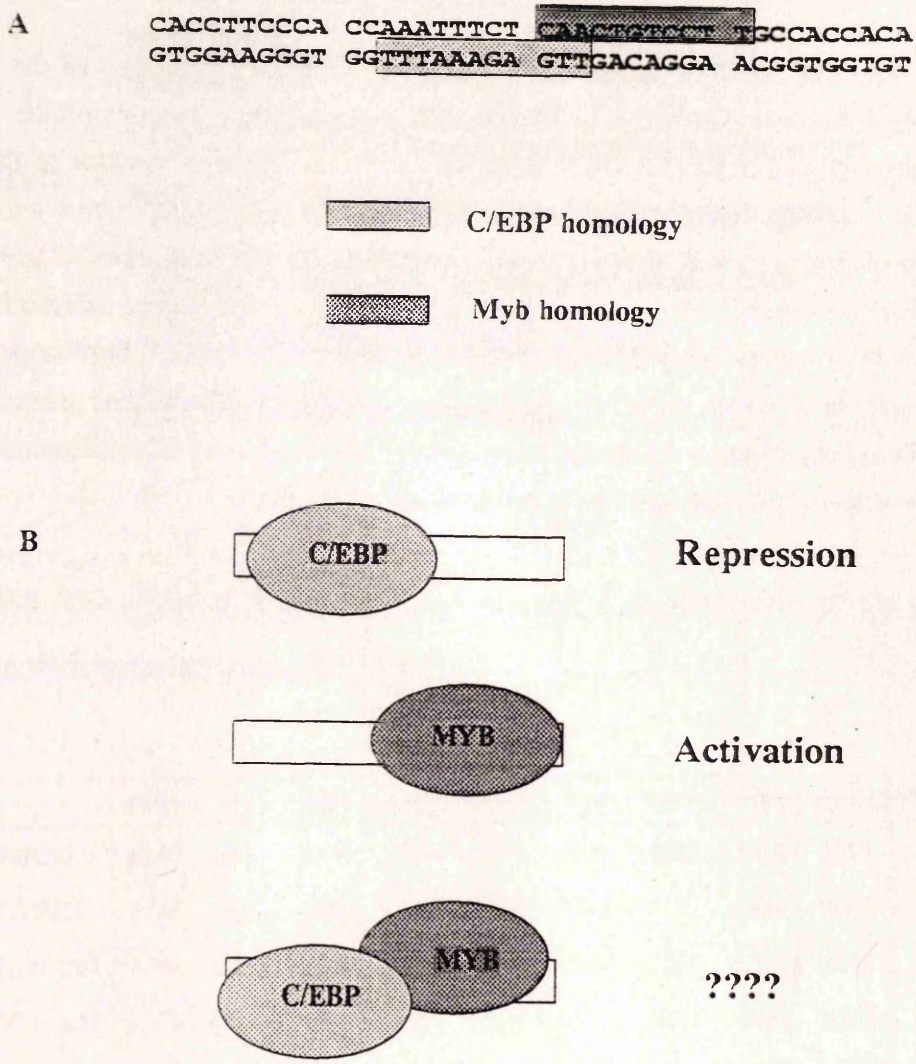


Figure 9.7 A: The FP1 sequence from the defensin promoter. Putative C/EBP and Myb binding sequences are illustrated by the shaded boxes. **B:** Potential protein-DNA interactions at the FP1 sequence. The possible consequences of these interactions on the promoter activity are indicated in the figure. This is discussed in detail in section 9.4 (i).

Figure 9.8: Histogram illustating the effect of exogenous c-*myb* expression on the activity of the pOGH-defensin promoter constructs in HL60 cells. Each bar represents the average activity from the results in table 9.8.

Table 9.8: The effect of exogenous c-*myb* expression on the activity of the pOGH-defensin promoter constructs in HL60 cells. An equimolar amount of the pOGH-defensin reporter construct (5μg for pOGH and an equimolar amount of the other constructs), 5μg of pSV2ASPAP, 5μg of pJ4myb or pJ4ΔmybR3 (where apropriate) and bluescript to a total of 50μg were cotransfected into HL60 cells as previously described in section 9.2. The medium was sampled 18 hours post transfection and 100μl was assayed for human growth hormone activity by radioimmunoassay as described in chapter 4.20(iii). The figures represent counts per minute after normalising transfection efficiency using the pSV2ASPAP as an internal standard. The average activity and standard deviation are shown in the table.

Figure 9.8: Illustrating the effect of exogenous c-*myb* expression on the activity of the pOGH-defensin promoter constructs in HL60 cells

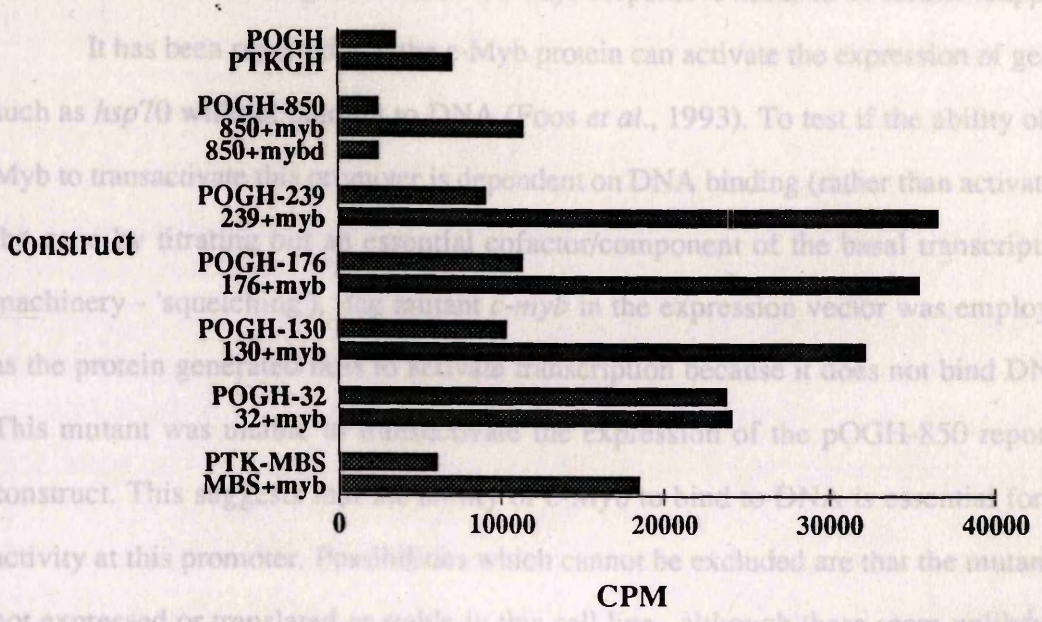


Table 9.8: The effect of exogenous c-*myb* expression on the activity of the pOGH-defensin promoter constructs in HL60 cells

	exp1	exp2	exp3	exp4	exp5	exp6	exp7	exp8	averag	std dev
POGH	5049	3176	4892	3060	2131	3221	4123	3076	3591	1006
PTKGH	6715	5122	9849	6157	5157	7664	8753	6994	7051	1661
POGH-850	3156	2233	1534	3080	1943	2790	3122	2315	2521	607
POGH-239	13725	10114	7894	4960	9768	8795	8893	8699	9106	2442
POGH-176	14132	11013	8943	11593	11042	10187	12376	11378	11333	1521
POGH-130	11978	9155	7768	15231	8543	7143	10687	12312	10352	2727
POGH-32	29460	32130	27485	11043	21031	23987	21087	24536	23844	6473
PTK-MBS	6551	7551	4556	5673	-	-	-	-	6082	1274
850+myb	13430	10699	4695	12134	10224	13451	15431	-	11437	3462
239+myb	34567	38675	-	-	-	-	-	-	36621	2904
176+myb	36823	34159	-	-	-	-	-	-	35491	1883
130+myb	37806	36272	27913	43189	27649	21002	31875	-	32241	7453
32+myb	31763	27312	27531	13476	19753	25611	23562	-	24144	5987
MBS+myb	18776	20114	18112	16943	-	-	-	-	18486	1323
850+mybd	3155	2422	1150	3753	2165	-	-	-	2529	991

myb expression. Clearly, however, a large amount of extra sequence is included in this construct and the fragment which is c-Myb responsive needs to be further mapped.

It has been reported that the c-Myb protein can activate the expression of genes such as *hsp*70 without binding to DNA (Foos *et al.*, 1993). To test if the ability of c-Myb to transactivate this promoter is dependent on DNA binding (rather than activating the gene by titrating out an essential cofactor/component of the basal transcription machinery - 'squelching'), the mutant *c-myb* in the expression vector was employed as the protein generated fails to activate transcription because it does not bind DNA. This mutant was unable to transactivate the expression of the pOGH-850 reporter construct. This suggests that the ability of c-Myb to bind to DNA is essential for its activity at this promoter. Possibilities which cannot be excluded are that the mutant is not expressed or translated or stable in this cell line, although these seem unlikely as both pJ4myb and pJ4mybΔR3 expression plasmids are identical except for an inactivating deletion in the DNA binding domain of *c-myb*.

9.4 (ii) c-Myb fails to transactivate the defensin gene in HeLa cells

To determine if the transactivation of the defensin promoter seen in HL60 cells could be repeated in non-haemopoietic cells, the above experiment was repeated in the epithelial cell line HeLa. However, in this case no transactivation of the pOGH-defensin promoter constructs could be seen using the *c-myb* expression vector (figure 9.9). The reasons for the lack of transactivation in HeLa cells is not clear, but there are a number of possible explanations. The first possibility is a failure to generate c-Myb in this cell line. The promoter should be active in this cell line, however, c-Myb itself may not be active, perhaps as a result of abnormal phosphorylation or rapid degradation. This is suggested by the inability of this construct to transactivate the synthetic c-Myb-responsive promoter (figure 9.9) which was increased 3-fold in the HL60 cell line in response to exogenous *c-myb* expression (figure 9.8). Other

Figure 9.9: Histogram illustating the effect of exogenous c-*myb* expression on the activity of the pOGH-defensin promoter constructs in HeLa cells. Each bar represents the average activity from the results in table 9.9.

Table 9.9: The effect of exogenous c-*myb* expression on the activity of the pOGH-defensin promoter constructs in HeLa cells. An equimolar amount of the pOGH-defensin reporter construct (5μg for pOGH and an equimolar amount of the other constructs), 5μg of pSV2ASPAP, 5μg of pJ4myb or pJ4ΔmybR3 (where apropriate) and bluescript to a total of 50μg were cotransfected into HeLa cells as previously described in section 9.2. The medium was sampled 18 hours post transfection and 100μl was assayed for human growth hormone activity by radioimmunoassay as described in chapter 4.20(iii). The figures represent counts per minute after normalising transfection efficiency using the pSV2ASPAP as an internal standard. The average activity and standard deviation are shown in the table.

Figure 9.9: Illustrating the effect of exogenous c-*myb* expression on the activity of the pOGH-defensin promoter constructs in HeLa cells

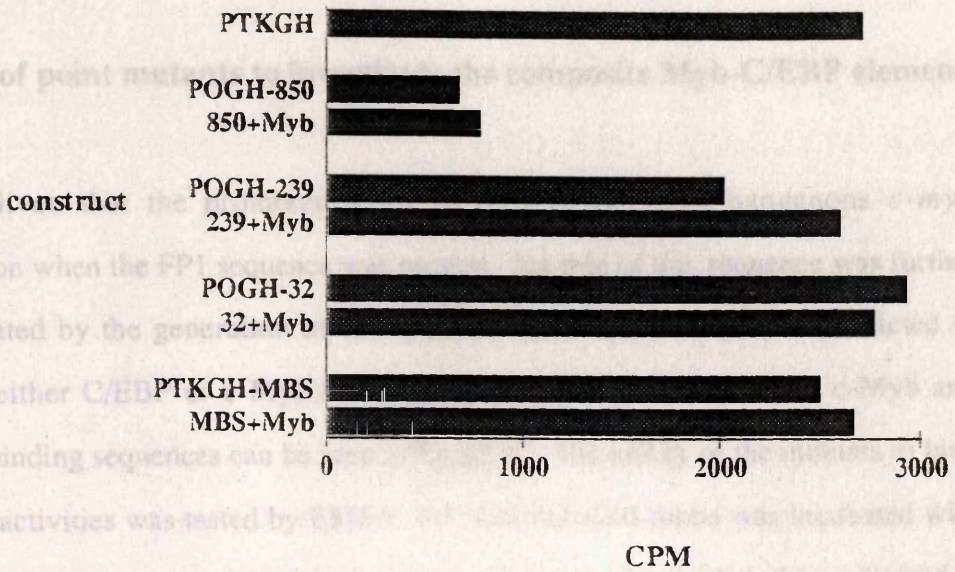


Table 9.9: The effect of exogenous c-*myb* expression on the activity of the pOGH-defensin promoter constructs in HeLa cells

	exp1	exp2	exp3	exp4	average	Std dev
PTKGH	2143	3187	3359	2179	2717	646
POGH-850	677	511	812	695	673	123
POGH-239	1534	2118	2674	1769	2023	495
POGH-32	2133	3124	4111	2378	2936	889
PTKGH-MBS	1892	3016	3190	1899	2499	700
850+Myb	813	512	917	892	783	186
239+Myb	1889	2512	2895	3128	2606	468
32+Myb	2234	3016	3379	2457	2771	521
MBS+Myb	2078	2917	3673	2017	2671	783

explanations such as the absence of another factor essential for promoter activity are possible.

9.5 Use of point mutants to investigate the composite Myb-C/EBP element

Given that the promoter could be transactivated by exogenous *c-myb* expression when the FP1 sequence was present, the role of this sequence was further investigated by the generation of FP1 point mutants which would be predicted to abolish either C/EBP or c-Myb binding (figure 9.10 A). The putative c-Myb and C/EBP binding sequences can be seen in figure 9.7. The ability of the mutants to bind the FP1 activities was tested by EMSA. FP1 radiolabelled probe was incubated with HL60 nuclear extract, and competitor oligonucleotides were included as indicated in figure 9.10B. This reveals that, as predicted, the mutant muC fails to compete with the wild-type sequence for binding of FP1 binding protein(s). The c-Myb binding mutant muM however also competes less efficiently for the FP1 DNA binding protein(s) than the wild-type sequence. Whether the FP1 muM mutant would bind c-Myb less efficiently than the wild type is unknown, as I was unable to translate *c-myb* RNA in a reticulocyte lysate (data not shown). Clearly this is an important experiment, and the approach of expressing this protein in bacteria could be undertaken. The point mutations however would be predicted to abolish binding as the C at nucleotide -109 (mutated to A) and the G at nucleotide -107 (mutated to C) were found in 51 and 46 out of a total of 51 potential c-Myb binding sites generated by the selection from random oligonucleotides (Howe and Watson 1991).

These mutant constructs were transiently transfected into HL60 cells to assay the effect of the mutations on both the basal activity in these cells and the ability to respond to exogenous *c-myb* expression. The results can be seen in figure 9.11. Contrary to expectations, the muC mutant shows a reduced level of activity compared to the wild-type construct. This is suprising as the wild-type FP1 sequence seems to act as a repressor when included in the promoter constructs. If C/EBP-like binding

Figure 9.10 A: Illustrating the sequence of the mutant promoters. The pOGH-130 contains the wild type sequence of the defensin HNP1A from -130 to +10. The constructs muM and muC are identical except for the point mutations which are indicated by the shaded boxes.

B: Investigation of the DNA binding properties of the mutant FP1 sequences. An EMSA was carried using radiolabelled FP1 oligonucleotide and HL60 nuclear extract as described in methods. HL60 nuclear extract (5μg) was included in the reactions as labelled in the figure. Competitor double-stranded oligonucleotides were added at 50-fold molar excess as indicated in the figure. The sequence of the sense strand of these mutant oligonucleotides are listed.

wt : AAGCTTACCAAATTTCTCAACTGTCCTAAGCTT

muC: AAGCTTACCAAAGGTATCAACTGTCCTAAGCTT

muM: AAGCTTACCAAATTTCTCAAATCTCCTAAGCTT

A

AAGCTTCCCACCAAATTTCTCAACTGTCCTTGCCACC POGH-130

AAGCTTCCCACCAAGGGTATCAACTGTCCTTGCCACC muC

AAGCTTCCCACCAAATTTCTCAAATCTCCTTGCCACC muM

□ - point mutant

B

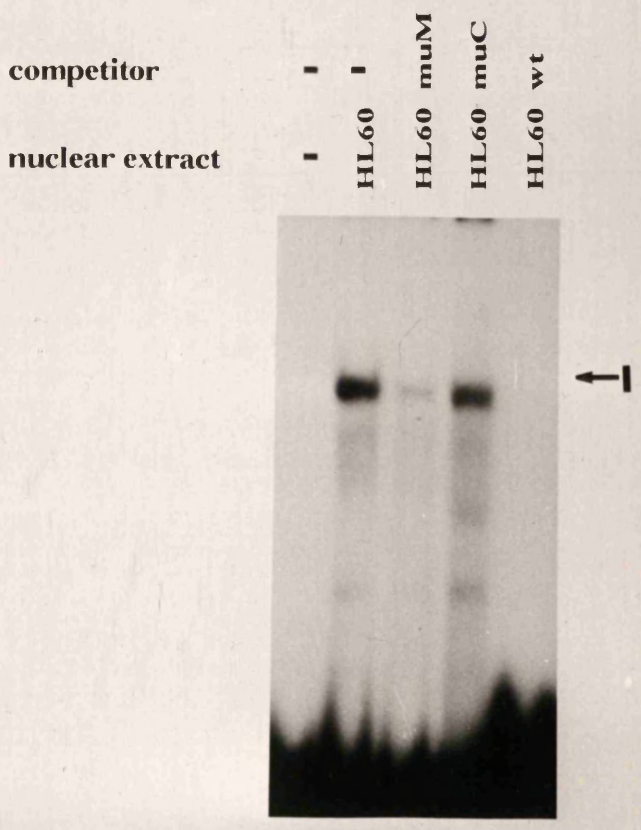


Figure 9.11: Histogram illustrating the effect of expression of exogenous c-*myb* on the FP1 mutant promoters in HL60 cells. Each bar represents the average activity of the results in table 9.11.

Table 9.11: The effect of exogenous c-*myb* expression on the human growth hormone activity of the FP1 mutant promoter constructs after transient transfection into HL60 cells. 5µg of the FP1 mutant promoters, 5µg of pSV2ASPAP, 5µg of pJ4myb (where apropriate) and Bluescript to a total of 50µg were cotransfected into HeLa cells as previously described in section 9.2. The medium was sampled 18 hours post transfection and 100µl was assayed for human growth hormone activity by radioimmunoassay as described in chapter 4.20(iii). The figures represent counts per minute after normalising transfection efficiency using the pSV2ASPAP as an internal standard. The average activity and standard deviation are shown in the table.

Figure 9.11: Illustrating the activity of the FP1 mutant promoters in HL60 cells and their response to exogenous *c-myb* expression

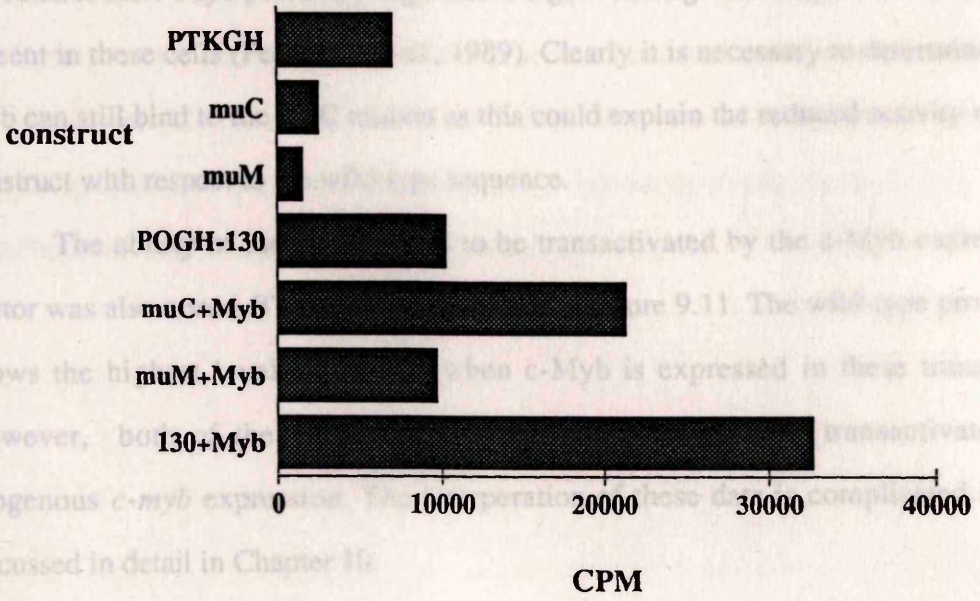


Table 9.11: Human growth hormone activity of the FP1 mutant promoters in transiently transfected HL60 cells and in response to exogenous *c-myb* expression

	1	2	3	4	5	6	7	8	averag	std dev
PTKGH	6178	9643	3987	7654	5432	8539	7859	7122	7051	1801
muC	3785	2342	2412	2442	1765	2764	2248	-	2536	625
muM	1238	1398	2187	1916	1018	1756	1176	-	1527	432
POGH-130	9155	8195	14237	12456	8828	9654	7561	12345	10303	2395
muC+Myb	23971	13048	19865	27566	22317	25164	17895	-	21403	4536
muM+Myb	8161	7979	14572	11571	8659	9123	7925	-	9712	2487
130+Myb	31984	33168	42106	35431	28563	31875	25438	-	32652	5278

activity at FP1 was responsible for this repression, we might expect the muC sequence to have a higher activity than the wild-type sequence. The muM construct also shows reduced activity with respect to the wild-type construct. This would be expected if the c-Myb positively regulates the gene through this sequence as c-Myb is present in these cells (Pedrazoli *et al.*, 1989). Clearly it is necessary to determine if c-Myb can still bind to the muC mutant as this could explain the reduced activity of this construct with respect to the wild-type sequence.

The ability of these constructs to be transactivated by the c-Myb expression vector was also tested. The results can be seen in figure 9.11. The wild-type promoter shows the highest level of activity when c-Myb is expressed in these transients. However, both of the mutant promoters muC and muM are transactivated by exogenous *c-myb* expression. The interperation of these data is complicated and is discussed in detail in Chapter 10.

9.6 Effect of differentiation on the activity of the constructs

In addition to the identification of myeloid-specific sequences, the identification of the sequences which control the differentiation-stage-specificity of the expression of this gene was of particular interest. The long-term aim of the project was to characterise the proteins that would mediate this specificity to provide an insight into the control of differentiation and how this process may be subverted in leukaemia. Most of the work in this study has employed the HL60 cell line as a model of myelopoiesis. The advantage of this cell line is that the cells can be readily induced to differentiate. As the FP1 activity was rapidly down-regulated during DMSO-induced differentiation the effect of this rapid loss of the putative repressor could be tested by transient assay after DMSO treatment. Unfortunately treatment of the cells with DMSO led to a reduction of the transient transfection efficiency which made this approach unpracticable. Not only was the efficiency of transfection significantly different between induced and uninduced cells, but also, there was concern that the cells into

which DNA entered were a subpopulation of cells. This necessitated the generation of stable cell lines containing the constructs, allowing the assay of activity over the relatively long differentiation time course. Although this laboratory had successfully generated stable transfectants using the neomycin resistance gene as a selectable marker (K. Ryan personal communication), this gene interferes with the differentiation of this cell line (von-Melcher and Housman 1988). The use of the marker histidinol dehydrogenase was employed as an alternative but variations in the sensitivity of the cells between experiments made this approach unsuccessful.

Part 4: Discussion

10.1 Investigation of defensin gene expression

(i) Survey of cell lines for defensin gene expression

Previous data provided strong evidence that the defensin genes were expressed only in myelocytes. In summary, expression has been detected in myelocytes in normal bone marrow by *in situ* hybridisation, the peripheral blood of CML and occasionally in ANLL patients (consistent with the presence of myelocytes in these samples, Daher *et al.*, 1989). In addition, expression has previously been reported in HL60 cells (Daher *et al.*, 1989;Wiedemann *et al.*, 1989).

The levels of defensin mRNA are relatively high in cells that express these genes (e.g. peripheral blood samples from CML patients consistently have very high levels despite consisting of a heterogeneous population of cell types, of which only a fraction consist of myelocytes), as these genes encode proteins which comprise a major component of granule protein in neutrophils. Clearly if a myeloid cell line was maturation arrested at a myelocyte-like stage of differentiation, if they were representative of their normal or leukaemic counterparts, then we would expect them to express the defensin genes. To date, no myelocyte like cell line has been isolated, presumably because this represents a relatively late stage of differentiation. HL60 cells however, are predominantly promyelocytic in nature. Expression of the defensin genes in these cells could be a result of some transcription occurring in the immediate precursor to the myelocyte. Alternatively, as the HL60 cell line is comprised of a heterogeneous population of cells, expression could be within a more mature sub-population. Although around 90% of HL60 cells are promyelocytic, the other 10% of the population are more mature cells which have/are spontaneously differentiating

(Collins *et al.*, 1977). Variation between different sub-lines can occur with earlier passages showing a greater propensity to spontaneously differentiate. This could explain why some lines express the defensin genes and others do not, however, it is unlikely to explain why the line in this laboratory ceased to express these genes. Analysis of 6 sub-lines of HL60 (Lamb 1990) suggested that expression of these genes is not common in this cell line. If the lack of expression of the defensin genes was a result of simply HL60 representing a more primitive cell, then induction of differentiation of this cell line would be expected to activate expression of this gene. Expression of these genes could not be detected and western blot analysis could detect no production of protein during a time course of induced differentiation of this cell line.

A number of other myeloid cell lines were also analysed for expression of the defensin genes. These cell lines appear to represent more primitive progenitors than the HL60 cell line. The logic of this approach was that if for some reason expression of the defensin genes was selectively disadvantageous then cells may evolve such that they are not expressed. However, if these more primitive cells can be induced to differentiate through the defensin expressing myelocyte stage then there would be no selective disadvantage to expressing these genes as the cells will be committed to terminal differentiation. However, again no expression of the defensin genes could be detected.

A large range of cell lines which might be predicted to express the defensin genes were tested for expression without success. There were two possible explanations. The first is that the defensin proteins cannot be tolerated (i.e. selectively disadvantageous) in tissue culture and hence cells rapidly evolve to switch off expression. The second is that the environment *in vitro* is such that the differentiation program/ myeloid phenotype is aberrant, perhaps due to lack of stromal contact or absence of soluble regulatory molecules. If the latter explanation is correct how can we explain the previous detection of defensin gene

expression in cell lines? One possibility is that the expression was the result of an inflammatory reaction, rather than a consequence of the differentiation status of the cells. Although the defensin proteins have been identified almost exclusively in neutrophils, they have been reported to be present in rabbit alveolar macrophages; in addition, the levels of these peptides were increased when the rabbits were challenged with the inflammatory agent Freunds Adjuvant (Lehrer *et al.*, 1981). Although defensin peptides have not been detected in human macrophages it is not inconceivable that under certain circumstances they may be induced to express the defensin genes. Along similar lines, an indirect effect of an inflammatory reaction could induce expression; a culture of HL60 cells contains a small amount of mature macrophage and neutrophil-like cells and these may respond to a pyrogen by the generation of cytokines such as IL-6 or G-CSF. These factors may induce differentiation or the activation of a subset of genes which are a component of the differentiation program. It is also possible that these factors may synergise with DMSO to induce a more representative differentiation program. These questions were addressed by treating cells with a combination of inflammatory and differentiation-inducing agents; however, no defensin either at mRNA or protein level could be detected.

A component of the serum used at the time may have an activity which potentiates differentiation and is necessary for the expression of the defensin genes. However, to test this possibility requires the comparison of batches of serum which are unobtainable. Clearly why the defensin genes are not expressed is a mystery, and any explanation must take into consideration that there are two different genes present in HL60 cells suggesting that direct mutation is an unlikely mechanism (as there are no gross deletions around the genes). One possibility investigated in this study was that methylation was responsible for repressing gene expression.

(ii) The role of methylation in the repression of defensin gene expression

Hypomethylation has long been correlated with gene expression and two classes of promoters can be identified with respect to methylation:

1) Genes that are CpG rich and are often constitutively unmethylated, generally a characteristic of housekeeping genes.

2) Genes which are methylated in all tissues in which they are not expressed; this methylation pattern is characteristic of tissue-specific genes.

The defensin genes appear to be an example of the latter, being methylated in all non-expressing cells tested, including a sample from a leukaemia patient which contained 90% myeloblasts, suggesting that in immature cells of the granulocytic lineage the defensin genes are also methylated and demethylation occurs during differentiation. The general problem in the interpretation of studies of this nature is that the data is correlative i.e. does the demethylation precede the transcription or is it a consequence? However, much emerging data suggests that methylation may play a key role in the regulation of gene expression. This was discussed in the Introduction in section 2.1(ii), and involves two potential mechanisms; namely, either the methylation of key transcription factor binding sites which prevents the binding of the appropriate factor, or the binding of repressor proteins specifically to methylated DNA. Evidence from a number of laboratories suggests that the second mechanism may be particularly important in the repression of CpG-rich promoters, and the inactivation of genes in tissue culture as a result of methylation of sites not normally methylated *in vivo* (Boyes and Bird 1991; Antequera *et al.*, 1991).

Are either of these mechanisms likely to be important in the repression of the defensin genes? As the defensin genes appear to be methylated in cells which

do not express the defensin genes *in vivo* (e.g. lymphocytes), whether the defensin genes are methylated in HL60 and as a consequence are not expressed, or simply are methylated as a result of not being expressed, is difficult to determine. If we postulate that these genes are repressed in HL60 by methylation, how is this to be mediated? As the defensin genes are highly expressed at the correct stage of differentiation one would predict that in these cells powerful promoter and/or enhancer elements would mediate this. It appears that the repression of transcription by the binding of proteins to mCpG is dependent on the density of mCpG surrounding the gene. A highly methylated gene is repressed in a transient transfection assay irrespective of the presence of a powerful enhancer. In contrast, a weakly methylated promoter is repressed but only in the absence of an enhancer (Boyes and Bird 1992). Analysis of the sequence available of the defensin HNP1A genomic clone reveals that this is not a particularly CpG dense gene and no CpG island has been identified. This would suggest that the binding of repressors to methylated DNA is unlikely to be the mechanism of repression in these cells. Alternatively, as there is a moderate number of CpG residues throughout the sequenced regions, and the HL60 genome appears to be grossly under-methylated in comparison to both that of the lymphocytes and all of the leukaemia samples studied (see section 6.6, figure 6.9), it may be that higher levels of mCpG binding proteins, MeCP1 or MeCP2 are available to mediate a repressive effect on those sequences which are methylated in this cell line (Boyes and Bird 1991).

Perhaps a more attractive model to explain the repression is the methylation of sequences which bind key transcription factors. The FP3 sequence mediates expression of reporter constructs in transient transfection in HL60 cells and appears to bind an Ets family member(s) (see section 8.5iii). The FP3 Ets-binding sequence CCGGA is methylated in HL60 cells. The result of methylation of this residue on the affinity of Ets family proteins for this sequence is unknown

but the binding of Ets-1 and Fli-1 to the almost identical E74 sequence requires a C immediately 5' of the GGAA sequence for high affinity binding (Fischer *et al.*, 1991; Zhang *et al.*, 1993). This suggests that the presence of a methyl group at this site could potentially prevent binding. Another area that may be an important region in the regulation of these genes is the first intron. This site has been found to contain a DNAse1 hypersensitive site in expressing cells suggesting it may be important in the regulation of these genes, although data on the factors which bind to this site remain to be elucidated. The first intron contains a high proportion of CpG residues in this region and hence could play a role in either preventing binding of transcription factors or may even bind to MeCP-like proteins preventing access of other transcription factors or altering the structure of the chromatin.

If the defensin genes are repressed by methylation then why is this repression not relieved by 5-azacytidine treatment? Obviously, this agent has a global effect on gene expression, as is apparent by its ability to induce differentiation. It is possible that although the defensin genes become demethylated as a result of the treatment, other changes within the cell transcriptional machinery may mean that the combination of transcription factors required for defensin gene expression is no longer present. This is not an unlikely scenario given that the defensin genes are expressed only during a very narrow window of differentiation, suggesting the specific regulatory regime for expression of these genes is very limited.

(iii) Why are the defensin genes not expressed in tissue culture?

Are the defensin genes not expressed because they are selectively disadvantageous to growth in culture? This is a possibility given the destructiveness of the defensin peptides to cells. Although these molecules are

sequestered to the granules and must be cleaved to become active, a small amount of leakage may be fatal. The HL60 cells have an incomplete differentiation program and do show some granule abnormality which may mean the defensins are not stored as well as they are *in vivo* (Newburger *et al.*, 1979; Olsson *et al.*, 1981). What effect the defensins would have within cells is unknown but in addition to the ability to puncture plasma membranes, they are very potent inhibitors of protein kinase C which may affect the growth and differentiation properties of the cells (Charp *et al.*, 1988).

As discussed previously, many genes are switched off in culture by methylation, presumably those which are not necessary for growth. The culturing of cells in high serum conditions places a high selective pressure for cells which can replicate more rapidly. The work of Bird and Boyes (1992), suggested that 50% of CpG islands were methylated in L and 3T3 cells indicating a massive switch off of non-essential gene expression. The loss of expression of genes which are non-essential for growth may include transcription factors which may play a role in generating the mature phenotype, and the early passage HL60 cell lines show a higher propensity to spontaneously differentiate than do the later passages. The loss of expression of the defensin genes may be a direct effect as a result of its non-essential nature and perhaps deleterious effects on growth. Alternatively, it may reflect an indirect mechanism such as the loss of a transcription factor which predisposes the cells to spontaneously differentiate which is involved in generating the mature phenotype. Loss of such a factor would be advantageous as the cells would have a selective advantage, but this loss may affect the pattern of induced differentiation as those genes which are regulated by this transcription factor will no longer be expressed.

As discussed in section 10.4, a C/EBP-like factor appears to play a key role in the regulation of defensin gene expression. One potential candidate is C/EBPβ, which has been implicated in the chicken to be involved in generating

the mature myeloid phenotype. If this is the case it may be that this factor can synergise with c-Myb on the defensin promoter to transactivate gene expression. Loss of this factor may generate a cell line less likely to differentiate spontaneously which would hence have a growth advantage (or alternatively, the activation of the negative regulator of C/EBPβ (LIP) activity could be up-regulated). In addition the C/EBP family can mediate growth arrest in heptatic and adipose tissue (Buck *et al.*, 1994). Clearly if a family member(s) mediates a similar effect in myeloid cells, loss of this factor's activity would be selectively advantageous for growth and possibly contribute to the imbalance between growth and differentiation that is manifested as maturation arrest seen in leukaemia.

10.2 DNAse1 hypersensitivity analysis

The DHS analysis was undertaken to provide some information as to areas of chromatin that may play some role in the control of defensin gene expression *in vivo*. A comparison was made between the chromatin structure around the defensin genes in both myeloid and non-myeloid cells and samples from both normal and CML samples. The CML samples were used as a source of defensin-expressing cells; this allowed us to assess if there were any sites which were ubiquitous, lineage-specific, differentiation-specific or correlated with expression of the gene. Clearly, interpretation of the results is not straightforward as a consequence of the CML samples being comprised of a mixed population of cells. Fortunately for this study, there seems to be no DHS in cells outside the myeloid compartment or in fully differentiated granulocytes which allows us to assume that the DHS identified in the CML samples are within the chromatin of the immature myeloid cells within these samples.

A summary of the DHS identified in this study are presented in figure 7.8. What does the presence of these hypersensitive sites tell us? As the sites were only found in myeloid cells and at a particular stage of differentiation, one could conclude that these sites could be generated by transcription factors that are restricted in activity to these cells (alternatively a combination of factors that are unique to these cells). Clearly identification of these factors would be of prime interest not just in regard to the regulation of the defensin genes, but to the regulation of myeloid-specific gene expression as myeloid-specific transcription factors (if they exist) have yet to be identified. In addition if these factors are only active in immature cells of the myeloid lineages, they could be candidates for activation in leukaemogenesis.

A question to be addressed is, what are the functions of these DNAse1 hypersensitive sites *in vivo*? It seems that all of the sites which have been studied in detail to date have an important biological function, and in addition to sites important in transcriptional control, have been implicated in occurring at sites of replication origins, attachment to the chromosomal matrix and topoisomerase II binding sites (Gross and Garrard 1988). As the defensin genes do not contain DHS over the region assessed outwith the myeloid compartment, it seems likely that the DHS identified was involved in the transcriptional control of these genes.

The difficulty with this study, is that the classical approach of footprinting these sites to identify any specific footprint is complicated by the mixed nature of the CML samples. It is possible to utilise mixed populations of cells and carry DHS analysis and compare the sites to differential cell counts, as if a specific cell type contains a DHS the intensity will be directly proportional to the number of these cells in the population. However the proportion of any given nuclear protein will not directly reflect the differential cell count as any DNA binding activity present could be the result of a small population of cells highly expressing the factor, or a large population of cells with low levels. As many

transcription factors can bind to sites *in vitro* which they never occupy *in vivo*, implicating a specific footprint in transcriptional control would be difficult.

10.3 The use of HL60 as a model system

A problem was faced in progressing this project. The DNAse1 analysis had suggested areas which may be important in mediating the transcriptional control of these genes, however, an extension of the work was problematical. One approach would be simply to characterise more CML samples (when they became available) for DHS to allow a correlation between particular sites and the proportion of certain cell types in the samples, allowing the identification of the stage of differentiation which the sites were hypersensitive. However, without a method of purifying particular cell types from these samples to allow characterisation of DHS by *in vitro* DNA binding studies, this approach would be of limited use. The use of mixed populations of cells in DNA binding studies is not desirable, even if the cell type of interest is in the majority. This is for two reasons, most obviously the source of DNA binding activity cannot be determined. Secondly, the nuclear extract taken from a sample does not represent a freeze frame of the transcription factor complexes present at the time of preparation of the extract. Rather, most transcription factors can dimerise and this can take place *in vitro*, often very rapidly. It is possible that complexes can be generated which do not exist in any of the cells present *in vivo*. A further disadvantage of the use of CML samples is that they cannot be employed in transfection assays. This is because the different cell types in the samples are likely to have very different transfection efficiencies, rendering any results unrepresentative (in addition, these samples could only be transfected at an extremely low efficiency).

To obtain the information which was the goal of the project i.e. the identification of transcription factors which mediate myeloid and differentiation-stage-specific expression, it was necessary to utilise a model human myelopoiesis. The HL60 cell line was employed as it would provide a number of useful advantages. Primarily this is a cloned cell line which hence can provide relatively large amounts of material for analysis. This cell line can be induced to differentiate with a number of different agents to either monocytic/macrophage or granulocyte like cells. This differentiation is well characterised and is accompanied by easily monitored changes in morphology, as well as by simple markers (reviewed in Collins 1987). This allows changes in the activity of transcription factors to be assessed using controlled experimental conditions, in addition the activity of reporter constructs can be assessed by introduction into this and other non-myeloid cell lines.

Clearly as this cell line does not express the defensin genes, this is not an ideal model. However, it is likely the lack of expression is the result of the loss of a single transcription factor, and as all genes are regulated by numerous DNA protein interactions, analysis of this promoter in these cells is likely to yield much information. In addition, as the aim of the project is to identify transcription factors which mediate myeloid and differentiation-specific expression then, if any reporter constructs show any myeloid-specificity or differentiation-stage-specificity in transfection assays, the identification of the transcription factors which mediate it is likely to be of interest, even if it does not represent the complete picture of the defensin transcriptional regulation.

For these reasons, the approach taken was to analyse the immediate promoter by DNA binding studies comparing nuclear protein from myeloid and non-myeloid cell lines and transfection assays of defensin promoter-reporter constructs in myeloid and non-myeloid cell lines. This allows a correlation to be

made between specific DNA binding activities and the ability to mediate transcription of reporter constructs.

10.3 (i) Transient versus stable transfectants

The analysis of promoter control regions cloned in front of a reporter gene can be undertaken using either stable or transient transfections. Each of these approaches has both advantages and disadvantages. The use of transient transfection has the major advantage of allowing rapid analysis of promoter constructs, but has a number of disadvantages. These include the introduction of many copies of a plasmid into a cell, which represents an unphysiological situation, as any cell will normally contain only two copies of a gene (for a single copy gene). This can lead to spurious results, in particular if a promoter sequence under analysis contains an element which binds a low abundance transcription factor. This effect of template being in excess can be seen if a titration is undertaken, where an increase in the amount of plasmid introduced into a cell will produce a corresponding increase in promoter activity only up to a certain concentration, when the level of a specific transcription factor(s) becomes limiting. To minimise this problem it is necessary to use the lowest concentration of template DNA possible in the transfection. In the transient transfections employed in this study 5μg of plasmid was typically used; however, in HL60 cells a relatively small difference was seen between the promoterless pOGH and the thymidine kinase promoter driven pTKGH plasmids. The small difference in activity between these constructs does not seem to be a result of saturation of the system as a reduction in the level of DNA does not increase the relative difference between them (data not shown). Similarly the activity of the SV40 promoter showed a linear increase in activity up to 20μg in these cells, suggesting that 5μg is not a high amount of template to be introduced into these cells, although the

exact level will vary between promoters. Previous reports of transfection into this cell line have reported the use of 5μg (Pahl *et al.*, 1991).

The other major problem with transient transfections is that the plasmids will not have the same chromatin structure as the endogenous gene, as although templates will be incorporated into core nucleosomes they may not have a 'positioned organisation' of nucleosomes into which the endogenous gene is organised. However in this case this may be an advantage as the endogenous defensin genes are not expressed in this cell line.

In addition to the above considerations, the method of transfection itself can have profound effects on cells. Electroporation causes considerable cell death and will elicit a range of responses including the activation of signal transduction pathways which will regulate transcription factors. An example of this has been documented, with transcription of the CD11b endogeneous gene being increased after electroporation of U937 cells (Pahl *et al.*, 1991). However, as this method was the only one available (in addition other methods of transcription themselves have problems e.g. calcium phosphate transfection could result in the activation of signal transduction pathways as calcium is an important second messenger) it was employed in these studies bearing these limitations in mind.

The use of stably transfected cells has a number of advantages over transients. The integration of the plasmid DNA into the chromosomes allows the assay of promoter activity of DNA within the context of chromatin, which can allow the assay of certain sequences which act to enhance transcription only when integrated into normal chromatin as they may have an effect in mediating chromatin structure (e.g. the Drosophila scs elements, cf. section 2.1). In addition, the copy number of plasmid templates will be low in comparison to a transiently transfected cell, as only a small fraction of those plasmids introduced will become integrated into the host cells' genome. The sensitivity of stable in comparison to transient transfections is much higher as almost all cells will

contain integrated plasmid. This greater sensitivity allows the direct analysis of reporter gene RNA and determination of the transcription start site if required. A further advantage of stable transfections is that they allow the effects of treatments to be measured over relatively long time periods, as for example the induction of differentiation of the HL60 cell line.

There are however, a number of drawbacks to the use of stable transfectants both practical and theoretical. Practically, the time-consuming nature of generating these cell lines is a limitation to their use. In addition, a number of considerations must be taken into account when stable transfections are employed. The DNA which is integrated is often in the form of concatamers; this could have a number of potential consequences which could generate unrepresentative results when analysing transcriptional control elements. The random integration of the DNA into different areas of the chromosomes subjects the promoter to position-dependent effects (see section 2.1) To control for this the use of a number of cloned stably transfected cell lines mixed together should be employed to obtain an average activity. Perhaps the most important consideration, when comparing the activity of a number of constructs, is to determine that the amount of DNA integrated into each of the cell lines is similar. This is because, broadly speaking, the expression of a gene will increase with increasing copy number. As a consequence, it is perhaps best to compare a range of constructs for relative activity using transient transfection, although a comparison using both methods would be ideal.

A study of the effect of differentiation on the activity of the reporter constructs during HL60 granulocytic differentiation would require the generation of stable cell lines. This is because although rapid changes could potentially be measured by transient transfection after DMSO treatment, this treatment significantly reduced the efficiency of transfection. The effect of other inducers of differentiation were not tested for their effect on the efficiency of transfection,

but it is possible that an agent such as retinoic acid may allow transfection to occur unimpeded. However, as the effect of this agent on the DNA binding activities identified in this study were unknown and the regulation of defensin gene expression was not identical during DMSO and retinoic acid induced differentiation (Lamb, 1990), this agent was not employed.

10.3 (ii) The transfection of myeloid cell lines

Myeloid cell lines are recognised as particularly difficult to transfect and electroporation seems to be the method of choice for transfection into these cell lines. However work in this study, and other published work suggests that this may be partially due to approach. It had been noted in a study by Pahl *et al.*, (1991), that the activity of reporter constructs rapidly diminished after 14 hours post transfection. The results in this work are in keeping with this finding, with no appreciable reporter gene activity detectable after 24 hour.

Perhaps the most important factor in determining the success of transfection into myeloid cell lines is the choice of reporter genes, with the use of secreted reporter genes being essential. A direct comparison between the sensitive luciferase assay and the secreted placental alkaline phosphatase (SPAP) reporter revealed that only the later could be detected. This result is not surprising given that the cytoplasm of these cells is full of degradative enzymes, and lysis of these cells will generate a particularly hostile environment for proteins which may prevent success of any reporter gene assay which involves the lysis of cells.

This interpretation is in keeping with the inability to detect β-galactosidase or CAT activity after transfection of plasmids expressing these reporter genes into HL60 cells, despite the electroporation protocol being successful at introducing DNA into these cells at a high enough level to detect both SPAP and secreted human growth hormone reporter genes. The use of secreted reporter genes may

be of general use in the transfection of other myeloid cell lines, as transfection of the mouse macrophage RAW cell line was only successful when the secreted reporter genes SPAP and human growth hormone were employed. Neither β-galactosidase nor CAT could be detected although a direct comparison was not made (Grove 1994).

Perhaps for a similar reason, the inclusion of excess non-specific plasmid DNA also increased the efficiency of transfection. In this case the activity of a nuclease may be responsible for degrading incoming DNA. The excess DNA may be acting as a competitor, although whether the excess DNA in fact stimulates greater up take of DNA cannot be eliminated. The reason for sonicated salmon sperm DNA being unable to increase the transfection efficiency is unclear but could be due to the putative nuclease being specific for supercoiled DNA.

10.4 Analysis of the defensin promoter

10.4 (i) What transcription factor(s) comprise the FP1 DNA binding activities?

As discussed in section 8.3 the FP1 binding activities in nuclear extract derived from HL60 cells seem to be comprised of C/EBP-like binding factors as:

-there is a recognisable C/EBP binding site within the FP1 sequence.

-point mutation of this putative site abolishes binding activity.

-the FP1 binding activities can be competed with the inclusion of excess C/EBP binding oligonucleotide in an EMSA.

-they are heat stable to 90°C.

If we presume that the activities are generated by C/EBP family members, what factors are likely to comprise it ? One possible candidate is C/EBPα, and this factor is expressed in HL60 cells. C/EBPα is present in uninduced cells and is down-regulated as the cells differentiate to either granulocytes or macrophage (Scott *et al.*, 1992). The western blot analysis in this study revealed two bands of similar size, which was interesting given that the FP1 activity is comprised of a doublet of similar mobility. If the FP1 binding activity was to be comprised of C/EBPα, then the slight differences in the time course of down-regulation (24 hours versus 8 hours) during DMSO differentiation could be explained by differences between the cell lines, or because the DNA binding activity of the protein (which is measured in this work) falls more rapidly than the protein levels (measured in the published study). C/EBPα is not present in normal macrophages so the presence of FP1-binding activity in TPA-treated HL60 cells could be explained as a reflection of the incomplete nature of the differentiation of this cell line if the FP1-binding activity was comprised of C/EBPα.

Other potential candidates to comprise the FP1 binding activity are C/EBPβ and C/EBPδ. There is no information as to the regulation of the C/EBPβ or C/EBPδ at the protein level in human myeloid cells, but in the mouse cell line 32D c13, differentiation toward granulocytes induced by G-CSF results in an up-regulation of C/EBPβ and a minor down-regulation of C/EBPδ (Scott *et al.*, 1992). If the situation in HL60 cells is analogous, this would suggest that the C/EBPα is most likely to comprise the FP1 binding activity as there appears to be no FP1 activity in fully differentiated granulocytes (8.3 (i)). We would perhaps expect that, if the HL60 cells were similar to the mouse 32D cells, there would be an up-regulation of C/EBPβ during granulocytic differentiation, however, no up-regulation of C/EBP consensus oligonucleotide binding activity could be detected by EMSA. It is possible to argue that, as the 32D cells are relatively immature, the up-regulation of C/EBPβ only occurs in progenitors as a

consequence of lineage commitment (towards the myeloid lineage) and in the more mature HL60 cells maximal levels have been achieved as a consequence of its relatively mature phenotype. The suggestion that C/EBPβ may be involved in lineage commitment is also suggested by work in chick cells, where the C/EBPβ homologue NF-M is rapidly induced in MEP cells when induced to differentiate along the myeloid lineage. As the changes of the C/EBPβ activity during HL60 differentiation are unknown, it is not possible to eliminate this factor as comprising the FP1-binding activity.

C/EBPβ is also regulated post-translationally and can be induced to translocate to the nucleus in response to TPA, kinases or LPS in chick macrophages (Katz *et al.*, 1993). The transient up regulation of the FP1 DNA-binding activity on TPA stimulation (8.4 (ii)) may be a reflection of this. Whether the human C/EBPβ can respond to TPA in this manner is unknown, but interestingly C/EBPβ is known to be directly regulated by IL-6 which has important effects (in addition to the regulation of acute phase proteins in the liver) on haemopoietic cells including stimulating the development of neutrophils and macrophage from GM-CFC (Metcalf 1989; Suda *et al.*, 1988). This IL-6 response is also conferred on heterodimers which could mean that any dimers containing this factor could mediate a response to IL-6 (Poli *et al.*, 1990). It will be of interest to determine if the suggested importance of C/EBPβ in differentiation is a result of its regulation by signal transduction pathways such as regulation by IL-6 and whether other differentiation signals such as G-CSF act through this factor.

Only limited work using EMSA with C/EBP-binding oligonucleotides with human myeloid cell nuclear extract has been published. One study utilised a fragment of the chick cMGF promoter which binds to NF-M. This suggested that in HL60 a single complex of low mobility bound to this sequence, and that in mature granulocytes there was no C/EBP activity but mature macrophages had

NF-M activity. This is a similar pattern to that of the FP1-binding activities and these workers suggest that these represent myeloid-specific novel factors on the basis of the sizes of cross-linked complexing experiments (Haas *et al.*, 1992). Whether this represents a novel C/EBP family member restricted to the monocytic compartment remains to be seen, but clearly the FP1-binding activity would be a candidate to comprise this NF-M activity.

A study of the expression of C/EBPα and NF-IL6 (C/EBPβ) in a number of myeloid cell lines at the mRNA level and by western blot has been published. In this study, C/EBPα mRNA, is slowly lost on TPA treatment of HL60 cells (lost by 12 hours) whereas it is rapidly lost on differentiation of the cells along the granulocytic pathway.This result would be in keeping with the FP1-binding activity being comprised of C/EBPα. Functional DNA-binding activity of C/EBPβ was measured using biotinylated oligonucleotide-capture and western blotting. This revealed two bands of similar mobility, and this active protein fell to a third of the maximal levels seen at 24 hours on differentiation of HL60 cells with TPA.

Clearly the data available suggest that any of C/EBPα, C/EBPβ, C/EBPδ or a novel factor could comprise the FP1 DNA-binding activities and the situation is further complicated by the ability of the C/EBP factors to dimerise (Poli *et al.*, 1990; William *et al.*, 1991; Cao *et al.*, 1991). As all of the C/EBP family dimers tested can homo- and hetero-dimerise we would perhaps expect a range of different C/EBP-binding complexes to be present in HL60 nuclear extracts, as this cell line co-expresses a number of family members. Since many of these dimers have similar affinities for a number of C/EBP binding sites (*in vitro*), we would perhaps expect the FP1-binding activities to represent a composite picture of all the C/EBP complexes present in the cells. Given that C/EBPα and C/EBPβ have different molecular weights (C/EBPβ and C/EBPδ have similar weights) we might expect a range of complexes of different

mobilities to be generated using a C/EBP-binding sequence and a HL60 nuclear extract in an EMSA assay. However, no significant difference in the size of the FP1-binding complexes can be detected.

Comparison between the pattern of retarded complexes generated using a symmetric C/EBP binding site and a FP1 oligonucleotide with HL60 nuclear extract in a EMSA reveals a very similar pattern, although they appear to be distinct. Comparing the patterns of retarded complexes generated during HL60 differentiation using these two oligonucleotides suggests that the FP1 oligonucleotide is binding a subset of those bound by the C/EBP oligonucleotide all of which have a similar mobility. As discussed in section 8.3(ii) the TPA induction results in a transient rise and a gradual reduction to basal levels of FP1-binding activity in the fully induced cell. In contrast, the C/EBP oligonucleotide binding levels in the fully induced cell are approximately 1/10 those seen in the uninduced cell. This result could be explained if certain C/EBP homo/heterodimers are showing differences in affinity between these two sites. Different homo/heterodimers can show differences in affinity for different C/EBP-binding sites, as has been demonstrated for the serum albumin site D (Roman et al., 1990).

To determine which factors are binding this sequence *in vitro* could be approached using the techniques of biotinylated oligonucleotide capture and western blotting and/or super shifting experiments using antibodies specific to the known C/EBP family members. Whether only a specific dimer binds *in vivo*, however is more difficult to determine.

10.4 (ii) What transcription factors comprise the FP2 DNA binding activities?

As discussed in section 8.4(iii), the DNA binding studies suggested that the FP2 binding activities had the properties of Ets transcription factors. The properties of these activities suggested that candidates to comprise them were PU.1 (Spi-1) and SpiB. This was suggested as:

-The relatively high mobility of the retarded complex generated in an EMSA using FP2 oligonucleotide and HL60 nuclear extract (Pahl *et al.*, 1992; Wasylyk *et al.*, 1992; Galson *et al.*, 1993).

-The tissue distribution of this activity as detected by gel shift analysis.

-The FP2 binding activities (using HL60 nuclear extract) could be competed by excess cold PU.1 sequence found in the SV40 promoter (Klemsz *et al.*, 1990).

-*In vitro* translated PU.1 has a similar mobility (in an EMSA when bound to FP2 oligonucleotide) as the FP2 complex I (8.4 (iv)).

Two complexes of similar mobility to these have been previously noted using extracts from HL60 and U937 cells when EMSAs have been undertaken using an Ets binding sequence from the CD11b promoter (Pahl *et al.*, 1992; 1993). The upper complex could be super shifted using antisera raised against PU.1, and *in vitro* translated PU.1 generates a similar mobility complex in an EMSA (Pahl *et al.*, 1992). It has been suggested that the lower complex is a degradation product as a result of a cleavage at the PEST sequence present in this protein. In this study the lower complex is not shifted by the addition of antibody; this would be predicted as cleavage at the PEST sequence would liberate a C-terminal sequence which contains a DNA binding domain lacking the peptide

against which the antibody was raised. The ability of N-terminal deletion mutants to bind DNA has been documented using *in vitro* translated PU.1 deletion constructs (Wasylyk *et al.*, 1992). Presuming the complexes represent PU.1 and a degradation product the question remains, does the degradation product represent degradation during the process of making nuclear extract or are both of these complexes present *in vivo*? The difficulty of making undegraded nuclear extracts from HL60 cells has been documented (Galson and Housmann 1989) and the B-cell line Haftl generates only a band with the mobility of the upper complex (Pahl *et al.*, 1992). It could be argued that the two complexes are a result of breakdown during the procedure as B-cells contain a lower concentration of proteases. However, it could be a reflection of the *in vivo* situation as the ratio of the two complexes appears to remain constant during differentiation; we would perhaps expect the ratio to be more variable if this degradation was a result of the isolation procedure. It has been suggested that protein interactions at the PEST sequence may protect the protein from degradation in Haftl cells (Pongubala *et al.*, 1992), suggesting that the cleavage may be regulated *in vivo*.

If the degradation product is present in similar amounts as the full length protein in myeloid cells what are the functional consequences? Perhaps the obvious one is the cells will contain proteins with functional DNA binding domains which may have lost the ability to transactivate gene expression. This might suggest that there will be competition for available sites. The loss of domains which may be involved in protein-protein contacts at particular promoters may also have important consequences. The ability of a truncated mutant of the Ets family to have profound effects on cellular processes has been documented. A truncated Ets-2 protein (lacking a transactivation domain) expressed under the control of an actin promoter could block CSF-1 dependent transformation of NIH 3T3 cells expressing the CSF-1 receptor (Langer *et al.*, 1992). Clearly it is difficult to speculate without further data, but one could

postulate that the truncated putative negative regulators could switch off a subset of PU.1 target genes (or other Ets family members targets), particularly as in this study it appears that these complexes do have separable DNA binding properties (8.4 iii).

It has not been determined conclusively that the identity of the FP2 DNA-binding activities are generated by the PU.1 transcription factor, and a number of the binding properties of these complexes could cast doubt on this proposition. Most obvious is the finding that the PU.1 binding oligonucleotide from the SV40 PU box does not compete as efficiently as the FP3 and E74 sequences which contain a C triplet 5' to the GGAA sequence (8.4(iii)). All of the previously identified elements which bind PU.1 contain a purine at these sites. This may suggest that the complex is not comprised of the PU.1 transcription factor (or alternatively, it may have its binding properties altered by post-translational modification in this cell type). However, *in vitro* translated PU.1 can bind to the FP2 site with high affinity. The closely related SpiB gene could be a candidate as it would be predicted to have a similar molecular weight and hence possibly generate a similar retarded complex (Ray *et al.*, 1992).

10.4 (iii) What transcription factors comprise the FP3 DNA binding activities?

Competition experiments have indicated that the FP3 binding activities can bind a number of Ets sequences. As discussed in section 8.5(iii), the high mobility complex iv (generated in an EMSA using HL60 nulcear extract and FP3 probe) seems to have very similar properties as upper complex I generated in a EMSA using the FP2 oligonucleotide and HL60 nuclear extract (section 10.4(i) 2). This complex can be competed by sequences capable of binding PU.1, and *in vitro* translated PU.1 generates a retarded complex with the same mobility as that

generated using HL60 extracts with FP3 probe. That the lower complex (perhaps cleaved PU.1) does not bind further suggests that the factors comprising FP2 complexes I and II have separable DNA binding properties.

The main complexes generated in an EMSA with HL60 nuclear extract and FP3 probe I, II and III are competed by only FP3 and E74 oligonucleotides (section 8.5 (iii)). These two oligonucleotides are similar in sequence and the E74 sequence which has been demonstrated to bind to Ets-1, Ets-2 and Fli-1/ErgB with high affinity (Klemsz *et al.*, 1990; Fischer *et al.*, 1991; Zhang *et al.*, 1993). Of these Ets-2 is expressed in the HL60 cells, and interestingly, a number of different transcripts can be detected (section 8.6). What the functional significance of these different transcripts are is unknown, but the generation of a number of complexes with the FP3 probe could be explained by postulating a number of different forms of Ets-2 proteins binding to this sequence. Alternatively, the complexes I, II and III could be generated by post-translational modification. Although Ets-2 is expressed in HL60 cells and is likely to bind this site with high affinity, the high level of the defensin-reporter gene pOGH-82 activity (which contains the FP3 sequence) in HL60 cells in comparison to Raji and HeLa would perhaps suggest that the transcription factor which mediates this expression has a restricted distribution (section 9.3). However, HL60 does not have the highest levels of Ets-2 mRNA (when compared to HeLa and Raji cell lines), and this factor has a widespread tissue distribution. It could be argued that the tissue-specificity seen in the transient assays is the result of the comparison to the HSV thymidine kinase promoter which could have different activities in these cell lines. This is suggested as the inclusion of the FP3 sequence (i.e. comparing activities of the pOGH-32 and pOGH-82 constructs in transient assays) does elevate transcription to some degree in these cell lines. However, it is significantly lower than that seen in HL60 cells (section 9.3).

The very large up-regulation of FP3-DNA binding activity in response to TPA treatment could be explained if these activities are comprised of Ets-2, which has been reported to be phosphorylated in response to this treatment or in response to growth factors (Bhat *et al.*, 1989 and 1990; Boulukos *et al.*, 1990). However, as the elevated activity of the pOGH-82 construct in HL60 cells (in comparison to HeLa and Raji cell lines) seems unlikely to be mediated by Ets-2, other candidates should be considered. One possibility is ErgB/Fli-1, which is expressed in HL60 cells and could potentially mediate tissue-specific gene expression given its restricted expression (Watson *et al.*, 1992). However, this factor generates complexes with significantly different mobilities in an EMSA (when translated in *vitro*) than the FP3 DNA binding activities present in HL60 nuclear extract, although it does bind with a high affinity to the FP3 sequence. In addition the FP3 binding activities seem to show a broad tissue distribution. It is possible that the FP3 binding activity detected in HL60 cells is comprised of Ets-2, given that this factor is expressed in these cells and can bind this sequence with high affinity. However, the myeloid specificity seen in the transient transfection experiments could be mediated by a separate transcription factor (such as ErgB), which may be low in abundance or difficult to detect under the conditions employed in the assay. If the activity mediated by the FP3 sequence (i.e. pOGH-82) in transients, is generated by the transcription factors detected in HL60 nuclear extracts by EMSA, then we would predict a large increase in reporter gene activity after introduction into HL60 cells and TPA treatment, given the very rapid and strong up-regulation of these binding activities as a result of this treatment.

The TPA induction of HL60 cells results in a very rapid increase in the activity of this factor. The rapid response suggests that this is perhaps a direct effect, perhaps by PKC-mediated phosphorylation of this factor. Whether this is involved in the induction of the differentiation program or is just a coincidental

TPA response is unknown. However, as a dominant negative mutant of Ets-2 can block CSF-1 mediated activity (Langer *et al.*, 1992), this suggests that genes regulated by Ets family members may be required for proliferation and differentiation within the monocytic compartment. The AP1 complex has also been implicated as a potential mediator of macrophage differentiation (e.g.Studzinski and Brevli 1987), and as Ets and AP1 often act in conjunction on some promoters (Wasylyk *et al.*, 1989 and 1990; Seth and Papas 1990), it could be that these factors acting together are important in committing cells to differentiate along this lineage.

10.4 (iv) The defensin gene is a target for c-Myb regulation

One of the most interesting results of the work is the suggestion that the defensin gene is a target of the product of the proto-oncogene c-Myb. To date very little is known about the targets of c-Myb, and although tentative evidence has implicated a number of genes the chicken *mim-1* is the only gene which has been conclusively demonstrated to be regulated by this protein. Identification of the targets of both the normal and oncogenically activated forms is likely to give an insight into the mechanisms by which this gene can contribute to leukaemogenesis and confer a specific differentiation phenotype.

The evidence in this study is suggestive in that reporter constructs can be transactivated in response to c-Myb and their expression is dependent on the presence of Myb binding sites. Transactivation by c-Myb can occur in the absence of DNA binding, as has been demonstrated for the activation of the *hsp*70 gene (Klempnauer *et al.*, 1989; Kanei-Ishii *et al.*, 1994). However, DNA binding is required for the transactivation of the reporter constructs as a mutant of c-Myb which cannot bind DNA fails to transactivate (section 9.4). Clearly this does not demonstrate that the endogenous gene is regulated by c-Myb as the conditions of a transient assay are somewhat removed from the normal *in vivo* situation.

The results produced using the point mutant of the FP1 sequence predicted to fail to bind c-Myb, are difficult to interpret. The mutant shows a reduced activity in HL60 cells, as would be predicted as these cells contain endogenous c-Myb. However, this mutant is still capable of transactivation by exogenous c-Myb. There are a number of possible explanations. The first is that the mutant promoter still binds to c-Myb. The reason for the reduced activity in HL60 cells could be explained by the reduced affinity of this sequence *in vitro*

for the FP1 binding activities. Hence this sequence could still be transactivated by c-Myb. The second possibility is that although c-Myb does not bind this mutant site with high affinity, the extremely high level of this factor generated by exogenous expression allows the reporter construct to be transactivated. A comparison of the response of mutant and wild type promoters to a titration of the c-Myb expression plasmid would be informative. A third possibility is that the transactivation by exogenous c-Myb is both a direct and indirect effect. The reduced activity of the mutant in HL60 cells is the result of a failure to bind to this sequence and the transactivation is the result of the regulation of the activity of a different factor. To distinguish between the possibilities it will be necessary to determine the relative affinities of c-Myb for these sequences *in vitro*.

The transcriptional control of the defensin genes and that of the *mim-1* gene are very similar. Both appear to be targets for c-Myb, a C/EBP factor is an important mediator of expression and binds at a composite Myb-C/EBP element in the defensin gene. Ets proteins could potentially be involved in the regulation of the defensin genes, and Ets-2 has been demonstrated to transactivate the *mim-1* promoter in transient assays synergistically with c-Myb (Dudek et al., 1992). Along with the similar restricted nature of the genes expression in both the chicken and human, the location of the protein in neutrophilic granules suggests that they may be homologues. A simple comparison carried out in this study revealed only limited homology, and the proteins are quite different in size. However T.Graf has evidence that the defensin genes are the human homologues of *mim-1* (personal communication).

Clearly if the defensin genes are the human counterparts of the chicken *mim-1* this adds weight to the proposition that the defensin genes are targets for regulation by c-Myb. It must be remembered that in this work only the defensin genomic clone HNP1A was analysed and evidence suggests that there are two defensin genes per haploid genome perhaps generated by the result of a recent

duplication and hence may be similarly regulated (Lamb 1990). However a third protein termed HNP4 , seems to be considerably diverged and its regulation during myelopoiesis is unknown (Wilde *et al.*, 1989).

As the c-Myb protein is required for proliferation in haemopoietic tissues it would perhaps be expected to have target genes which are involved in the cell cycle or proliferation. It seems unlikely that the defensin gene has a functional role in promoting proliferation or in the maturation arrest seen in CML patients myelopoiesis. Although it has been demonstrated that the defensins are mitogenic for fibroblasts and epithelial cells, this is probably to generate repair at wound sites and a role of this gene in transformation seems unlikely.

In addition to the role of c-Myb in proliferation, it has been suggested that this protein plays an essential role in differentiation. Evidence of this is discussed in section 3.4, but it is relevant to note that c-Myb is required for induced differentiation of HL60 along the granulocytic pathway but not the monocytic pathway, perhaps suggesting that 'c-Myb conditioned proliferation' is necessary to acquire a granulocytic phenotype (Ferrari *et al.*, 1990). As the defensin genes are an important component of the granulocytic phenotype, they may be one of a number of target genes regulated by c-Myb which are involved in generating a specific differentiation phenotype.

10.4 (v) Mechanisms of the regulation of differentiation-stage-specific expression of the defensin genes

The defensin genes are expressed during a narrow window of differentiation in the myeloid compartment. Given the data obtained in this study by transfection analysis and DNA-protein interaction studies on the promoter, it is possible to postulate attractive hypothesis to explain how the expression of these genes are controlled during differentiation. A number of assumptions

(which may not be correct - discussed later) must be made, the first of which is that the differentiation-stage-specific expression is mediated by the action of DNA-protein interactions taking place at the immediate promoter (i.e. the first 250bp). Other DHS sites are present in the CML samples so other regions may be involved in some aspects of transcriptional control. The second assumption is that HL60 is a model cell line which reflects the regulation that takes place *in vivo*; clearly some differences must exist due to the lack of expression in this cell line. If however the lack of expression is the result of methylation as a result of propagation in tissue culture then the results may be more representative of the *in vivo* situation. Bearing in mind these caveats the data seem to suggest mechanisms which may be relevant to the regulation of these genes *in vivo*.

Three transcription factors/DNA binding activities are likely to be of key importance, due to their ability to transactivate/repress expression of the reporter constructs and the modulation of their levels/ activity during differentiation. These are:

1) c-Myb
2) FP1 (C/EBP)
3) FP3 (Ets)

Evidence implicating c-Myb in transactivating the defensin promoter has been discussed in the previous section. This factor is down-regulated during HL60 differentiation, although the levels of protein are not completely depleted until late in differentiation of the HL60 cells (Pedrazzoli *et al.*, 1989) and activity of this protein is necessary for the granulocytic differentiation of HL60 cells as this is blocked by antisense treatment of these cells (Ferrari *et al.*, 1990).

The FP1 DNA binding activities are rapidly down-regulated during granulocytic differentiation and FP3 is present until late in the granulocytic differentiation pathway. A diagrammatic representation of the changes in DNA binding activities during HL60 differentiation is shown in fig 10.1.

Two basic hypothesis can be put forward to incorporate the data into a model explaining the differentiation-stage-specific regulation of defensin gene expression (illustrated in figure 10.2). The first makes the assumption that the FP1 activity is acting as a repressor, as the inclusion of this sequence results in down regulation of the reporter gene activity. The alternative explanation is that the FP1 binding activity is a positive regulator of transcription which rather than repressing reporter gene activity, redirects transcription through the 5' TATA element. Considering the first hypothesis, if the FP1 binding activity is acting as a repressor, then the activity of the defensin gene could be explained as occurring only in the absence of the FP1 binding factors. This repression could be both direct and indirect in that it reduces the activity of the promoter via a positive action and in addition it may function to prevent the binding of c-Myb to a site which is juxtapositioned (refer to figure 9.7). As the C/EBP family members are present in immature myeloid cells (Scott *et al.*, 1992; Ness *et al.*, 1993), this sequence may mediate repression of the defensin genes in cells less mature than myelocytes. Loss of this activity during granulocytic differentiation of HL60 cells occurs by 8 hours, allowing expression of the defensin genes to be positively activated by the FP3 binding activities (this sequence mediates high level expression in reporter constructs) and c-Myb which can transactivate these constructs. As the FP1 binding activity is not lost during monocytic differentiation of HL60 cells this could explain why the defensin genes are not expressed in this lineage.

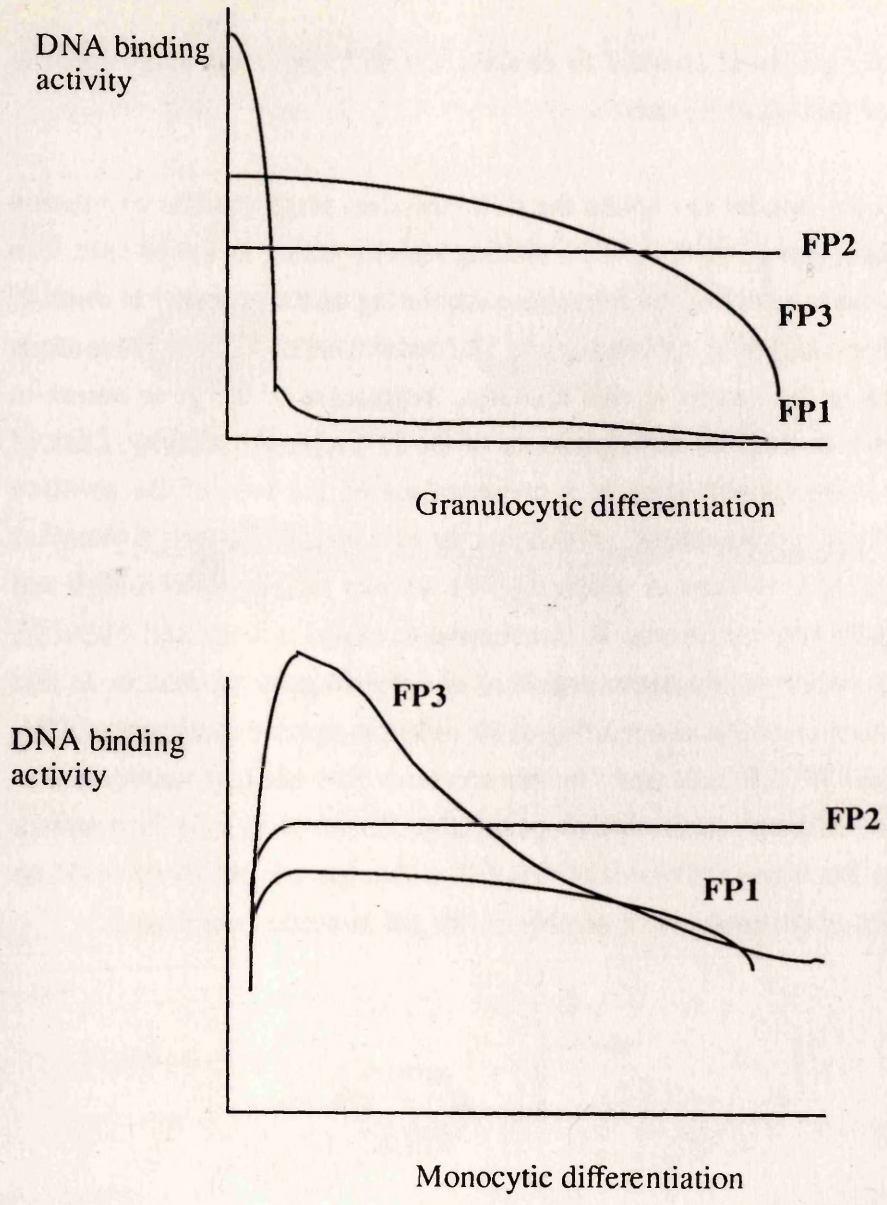
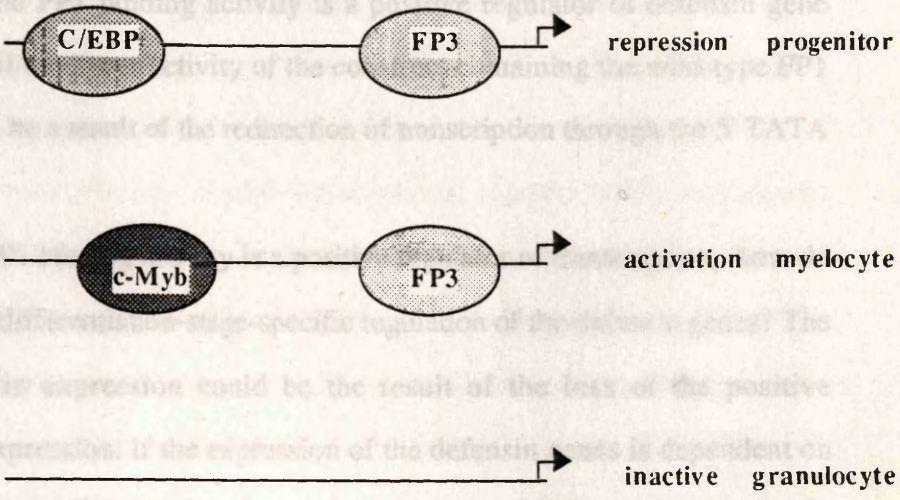


Figure 10.1 : Changes in the FP1, FP2 and FP3 DNA binding activity during HL60 differentiation. The DNA binding activity scale is arbitrary as the absolute levels of these activities with respect to each other are unknown. The granulocytic differentiation is induced by DMSO and monocytic differentiation by TPA. The granulocytic differentiation is completed by 5 days and the monocytic by 3 days after the addition of the appropriate inducer.

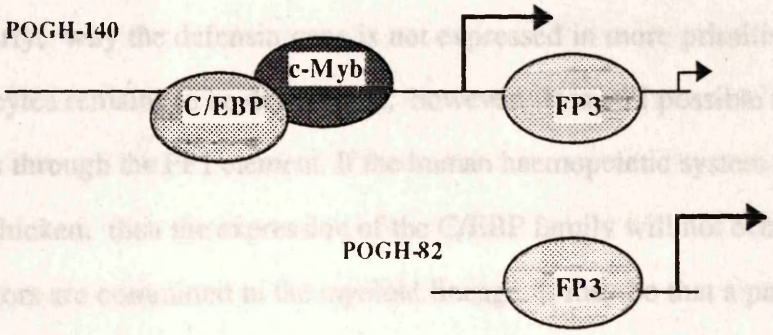
Figure 10.2 : Potential models to explain the differentiation-stage-specific expression of the defensin genes

Figure 10.2 A: Model to explain the differentiation stage specific expression of the defensin genes, with the FP1 binding activity acting as a repressor. The cartoons illustrate protein-DNA interactions occurring on the promoter in myeloid cells at different stages of differentiation. The consequences of these interactions are indicated in the figure. In this scenario, repression of the gene occurs in immature cells as a result of the presence of the FP1 repressor activity. Lack of expression in the granulocyte is a consequence of the loss of the positive mediators of this genes activity, c-Myb and the FP3 binding activity. Expression occurs in during a window in which the FP1 activity is absent and c-Myb and FP3 binding activity are present. **B:** An alternative model is illustrated where the FP1 binding activity is a positive regulator of defensin gene expression. In this figure, potential interactions occurring at the defensin-reporter constructs pOGH-82 and pOGH-140 are indicated. In this scenario, FP1 binding activity and c-Myb together activate transcription positively. However at least in transient transfections the lower activity of pOGH-140 with respect to pOGH-82 could be a consequence of the attempted assembly of two pre-initiation complexes.

A


C/EBP
FP3
repression progenitor
c-Myb
FP3
activation myelocyte
inactive granulocyte

B


POGH-140
c-Myb
C/EBP
FP3
POGH-82
FP3

Although this is an attractive hypothesis, the results from transfection with the point mutants suggest that the FP1 binding activity does not repress transcription of this gene. The mutant construct muC, which binds to the FP1 with a lower affinity than the wild-type sequence, has a reduced activity.This suggests that the FP1 binding activity is a positive regulator of defensin gene expression, and the lower activity of the construct containing the wild-type FP1 sequence could be a result of the redirection of transcription through the 5' TATA box.

If the FP1 binding activity is a positive regulator of transcription, how do we explain the differentiation-stage-specific regulation of the defensin genes? The loss of defensin expression could be the result of the loss of the positive activators of expression. If the expression of the defensin genes is dependent on the presence of the FP1 binding factor then the lack of defensin expression in mature cells of this lineage could be the result of the loss of this factor during granulocytic differentiation. In addition, the positive regulators of the reporter constructs FP3 and c-Myb are not active in mature granulocytes.

Clearly, why the defensin gene is not expressed in more primitive cells than myelocytes remains to be determined, however, it is still possible that the regulation is through the FP1 element. If the human haemopoietic system reflects that of the chicken, then the expression of the C/EBP family will not occur until the progenitors are committed to the myeloid lineage. It may be that a particular pattern of C/EBP family expression will modulate the activity of the FP1 factor(s). For example, the protein LIP which is generated by translation beginning at an internal AUG of the C/EBPβ transcript (Descombes and Schibler 1991), and negatively regulates C/EBP activity in sub-stoichiometric amounts, could control FP1 activity. This factor modulates C/EBP activity during adipose differentiation in mice, although whether a similar factor is generated in human myeloid cells remains to be seen. Similarly, why the defensin genes are not

expressed in monocytes is also unknown as all of the positive regulators appear to be present during HL60 granulocytic differentiation, although c-Myb is not present in mature monocytes. It may be that the activity of c-Myb is rapidly switched off during monocytic differentiation perhaps by regulation of phosphorylation, as this factor is not required for monocytic differentiation but is essential for granulocytic (Ferrari *et al.*, 1990). Alternative explanations are possible, such as sequences not analysed could be important mediators of defensin expression. The role of post-transcriptional control of defensin message could play an important role in the both the differentiation-stage-specific and lineage-specific (monocytic versus granulocytic) expression as the defensin message is relatively stable, suggesting that destabilisation of the message could be important in the reduction of defensin message levels (Lamb 1990). Clearly it would be interesting to analyse transcription rates during induced differentiation of HL60 cells with TPA and DMSO by nuclear run-on experiments. However as a defensin-expressing line of HL60 could not be found, this approach was not possible.

Although the evidence suggesting that the FP1 binding factor(s) is a positive regulator of defensin expression there are a number of caveats. The first is that the relative affinities of c-Myb for the mutant and wild-type promoters are not known, hence it is possible that the reduced activity of the muC mutant in HL60 cells could be the result of a reduced affinity for c-Myb rather than for the FP1 binding factor(s). A second consideration if FP1 sequence is directing transcription through the 5' TATA sequence is whether this represents the situation *in vivo* ? Although, both transcription start sites are employed in CML cells, the 3' site is clearly the major site of transcriptional initiation (Lamb 1990). It may be that the transient assay gives an artefactual result because it allow the attempted assembly of two pre-initiation complexes (given the relatively relaxed state of the chromatin in the assay), which would not happen in the normal

chromatin context. Hence it may be that if stable transfected cell lines were tested the inclusion of this sequence may up-regulate the expression of the reporter gene further.

What role does this second transcription start site play *in vivo* ? The functional consequences of the different transcripts are unknown, but could affect the stability of the message. It is possible that the 5' TATA box may direct transcription of the gene in response to a separate set of transcription factors. The identification of defensin proteins in rabbit alveoli macrophages after immune challenge, indicate that these genes may be expressed in cells other than myelocytes. C/EBPβ and C/EBPδ can have their activity mediated by inflammation (Poli *et al.*, 1991), and could direct transcription through this second start site as a result of binding to the FP1 sequence. These possibilities are highly speculative, and it will be important to determine transcriptional start sites in stably transfected cell lines to gain further insight into how these sequences are mediating their effects.

10.4 (vi) The defensin genes and myeloid-specific gene expression

Transcription factors which are limited strictly to the myeloid lineage remain to be identified (if they exist). A limited number of genes whose expression is restricted to the myeloid compartment have been studied to determine the transcription factors which mediate this specificity. If there are no myeloid-specific transcription factors, then how is this lineage-specific expression achieved? The CD11b gene, which is expressed only in myelomonocytic cells, has been demonstrated to show myeloid-specific expression and the transcription factor PU.1 is necessary for this activity (Pahl *et al.*, 1992). However, this is not myeloid-specific in the sense that only the cell lines U937 and HeLa were compared. As the PU.1 gene is expressed in B-cells

also (Klemsz et al., 1990), whether this promoter would be transactivated in these cells is unknown, suggesting that other sequences are likely to be important in this process. Many genes can show an elevated expression when reporter constructs are introduced into an expressing rather than a non-expressing cell line, but this is often only reflects a component of the tissue-specific regulation of the endogenous genes. The study of liver-specific gene expression liver may provide a model for the tissue-specific expression of myeloid genes, as no transcription factor which is strictly limited to the liver has been identified. Intensive study of a number of genes has suggested that the combination of a number of elements which bind transcription factors, which are expressed at elevated levels but not restricted to the liver, together generate a liver-specific promoter or enhancer (Rouet *et al.*, 1992; reviewed in Dai and Darnell 1992).

The data from the transfection experiments (Chapter 9) suggest that the promoter is myeloid-specific, although it must be remembered that only HL60 , Raji and HeLa cells have been tested using transient assays. Other sequences may be important in the myeloid specificity of this gene, as the effect of more distal sequences may be obscured by the repressive effect of the FP1 sequence. Linker scanning mutation would be necessary to determine if these sequences make a contribution. The possibility that other elements are likely to be important in the transcriptional control of these genes is suggested by the detection of 5' DNAse1 hypersensitive sites elsewhere (Chapter 7). Although there may be other elements involved in the generation of myeloid specificity, the analysis of the transcription factors which elevate expression in HL60 relative to HeLa is likely to be informative. The inclusion of the FP3 binding sequence elevates the level of transcription with respect to that seen in HeLa or Raji cells (although in HeLa some weak activity is generated). This result suggests that this activity is mediated by a factor absent (or inactive) or present at reduced levels in Raji and HeLa with respect to HL60 cells. As discussed in section 8.5 this sequence binds

an Ets-like activity(s), and could be comprised of the transcription factor Ets-2. This factor however, has a relatively broad tissue distribution, and is expressed at a higher level in the HeLa cell line than in the HL60. Hence it may be unlikely that Ets-2 is mediating this elevated expression in the transient transfection experiments into these cell lines. It may be that another factor, which is less abundant or undetectable by gel EMSA using these conditions, may be mediating this response rather than the FP3 binding activities detected. ErgB, which has a limited tissue distribution, and is expressed in HL60 cells (Watson *et al.*, 1992), could be responsible for the elevated expression of the constructs in HL60 cells. This factor can bind to the FP3 site with high affinity when translated *in vitro* and hence may play a role *in vivo*.

The constructs show some up-regulation in HeLa cells when the FP3 sequence is included, and this weak activity may be generated by Ets-2 which may bind this sequence in these transient assays but perhaps not the endogenous gene. An alternative explanation is that the levels of active Ets-2 are relatively higher in HL60 cells than in HeLa cells, or interaction with another factor which is present only in the HL60 cells is required for Ets-2 to be active on this promoter. It is interesting to note that the *mim-1* gene could be transactivated by expression of Ets-2 synergistically with c-Myb (Dudek *et al.*, 1992). Whatever the Ets family member responsible for this activity, it seems likely that this it will contribute to the tissue-specificity of the promoter.

The second site of protein-DNA interactions which are of interest in terms of myeloid specificity is the FP1 sequence. This element binds to a C/EBP like factor and c-Myb. Constructs which contain this element can be transactivated by c-Myb, suggesting that c-Myb may be important in the expression of the defensin genes. C-Myb is restricted to the haemopoietic lineage and has been suggested to generate a combinatorial myeloid-specific signal in conjunction with C/EBP (Ness *et al.*, 1993; Burke *et al.*, 1993). Although in this study it is not

clear whether C/EBP is acting as a repressor or as an activator, both scenarios would have important implications for the myeloid-specific regulation of this gene. If the FP1 binding activities are positive regulators of defensin expression, then the myeloid-specific expression of the defensin genes can be explained similarly to the *mim-1* gene with C/EBP and c-Myb providing a myeloid-specific signal as this lineage appears to be the only one in which these two factors are co-expressed. If the FP1 binding factor(s) is a repressor of defensin expression, then this activity may be important in preventing expression in the monocytic compartment. As all of the DNA binding activities found to date which may be positive regulators of the defensin gene expression are also present in HL60 cells that are differentiating along the monocytic pathway (i.e. FP2, FP3 and c-Myb). The defensin genes are not expressed during monocytic differentiation and this may be a result of the continued presence of the FP1 DNA binding activities repressing activity in this pathway.

Does C/EBP have a role in repressing/activating gene expression in other tissues of the haemopoietic compartment? Evidence suggests that there is no C/EBP activity outside the myeloid compartment and that is in keeping with the data here, although this is purely circumstantial and a detailed analysis of C/EBP expression by northern blot has not been undertaken. The reason for the lack of expression of the defensin genes in erythroid cells (if C/EBP acts as a repressor and is absent) could be due to the lack of expression of the Ets family members such as ErgB/Fli-1, which may not be expressed in erythroid cells (Watson *et al.*, 1992). However, the activity of the defensin-reporter constructs have not been tested in an erythroid cell line.

The effect of the FP2 sequence in the transient transfections may be obscured by the negative effect of the FP1 sequence, but the DNA binding data suggests that this sequence may bind PU.1 (Spi-1) (see section 8.4iv). As this protein is present in HL60 cells (Watson *et al.*, 1992; Pahl *et al*, 1992), it may

also be involved in the myeloid-specific expression of the defensin genes, although this factor is not strictly limited to cells in the myeloid compartment, being expressed also in B-cells and at low levels in CFU-E and mast cells (Klemsz *et al.*, 1990; Galson *et al.*, 1993b).

Although there is no real data to support the proposition, it is possible that methylation could play a key role in the tissue-specific expression of the defensin genes (or the differentiation-stage-specific expression). This is because the gene is methylated in immature cells which do not yet express the gene (and non-expressing tissues). However, as discussed in section 6.7, this could simply reflect demethylation as a consequence of transcription. Demethylation of specific sequences has been shown to be important in the tissue-specific regulation of a number of genes, including the gene encoding histone H2b in which demethylation of specific sites is involved in testis-specific transcription (Choi and Chae 1991). Stage-specificity has been demonstrated to be mediated by demethylation of a single CpG residue in a control region of the mouse M-lysozyme gene. A single CpG residue in a 3' enhancer of this gene is methylated in immature macrophages, and demethylation of this site with differentiation allows the binding of a ubiquitous transcription factor and transcriptional activation (Klages *et al.*, 1992). As the FP3 sequence is capable of binding to a number of factors (as tested by EMSA using nuclear extracts from a range of sources), it may be that methylation of this sequence contributes to repression in inappropriate tissues *in vivo*.

10.5 (i) General conclusions

The aim of this project was to use the defensin gene as a model which because of its restricted expression pattern, could be studied to identify transcription factors which may be involved in myeloid-specific and/or

differentiation-stage-specific gene regulation. This was undertaken with the general aim of understanding both the normal control of myelopoiesis and how this may be altered to generate maturation arrest and leukaemia. Of particular interest would be transcription factors whose activities are rapidly modulated on the relief of this maturation arrest by the induction of differentiation, as this may indicate factors whose down/up-regulation is necessary for differentiation to occur. How successful has this study been in contributing to answering these questions? A number of transcription factors have been implicated in the control of this gene. Of these, c-Myb is a target for leukaemic transformation and has an essential role in both proliferation and differentiation in the haemopoietic system. Work from a number of laboratories has implicated c-Myb as an important mediator of the myeloid phenotype. They suggest that c-Myb generates this myeloid-specificity by interaction with a C/EBP family member. A similar interaction is seen on the defensin promoter, suggesting that this 'combinatorial myeloid-specific signal' seen in the chicken system may have parallels in the control of human myelopoiesis. In this work, the FP1 binding activities (C/EBP-like) are lost rapidly on HL60 granulocytic differentiation and C/EBP factors are more slowly down-regulated. This and the involvement of these factors in differentiation control in other systems, begs the question, is the C/EBP family a potential target for leukaemic transformation and an involvement in maturation arrest? No data are available suggesting alteration in C/EBP family members in leukaemia. However a translocation present in many myxoid liposarcomas have been demonstrated in a number of patients to rearrange one of the CHOP/GADD153 alleles (Aman *et al.*, 1992). This implicates the alteration of this gene in the development of these cancers. Perhaps by altering the activity of the C/EBP family of transcription factors and blocking differentiation, as this factor is induced on differentiation of 3T3 blasts to adipocytes and is believed to be a negative regulator of C/EBP activity (Ron and Habener 1992).

The Ets transcription factors which comprise the FP2 and FP3 binding activities have not been conclusively identified; however, candidates are PU.1, Ets-2 and ErgB. The mouse homologues of PU.1 and ErgB, Spi-1 and Fli-1, respectively, are both involved in virally induced erythroleukaemia as a result of insertional activation (Bear *et al.*, 1989; Seth *et al.*, 1992). These transcription-factors, when over-expressed, may block erythroid differentiation: Spi-1 is over expressed in mouse erythroleukaemia cells and is down-regulated on DMSO induced differentiation of these cells (Galson *et al.*, 1993). Ets-1 and Ets-2 transform fibroblasts when they are over-expressed and are located (as are other Ets genes) near sites which are often translocated in leukaemia (reviewed in Papas *et al.*, 1990), though they have yet to be demonstrated to play a causal role in human leukaemia. The one *bona fide* example of involvement of an *ets* gene in human cancer is a characteristic reciprocal translocation between 11q24 and 22q12 seen in Ewing's sarcoma. This translocation fuses a portion of the *fli*-1 gene to a putative RNA binding protein which results in ectopic expression of the chimeric gene (Delattre *et al.*, 1992). Clearly the Ets family of transcription factors merit further investigation to determine what role they may play in the generation of human leukaemia.

An interesting observation of this study is the rapid changes in DNA binding activity of FP1 and FP3 binding proteins on differentiation, in a lineage-specific manner. That is, the FP1 binding activity is down-regulated on granulocytic differentiation, but remains relatively unchanged on monocytic differentiation, whereas the FP3 binding activity is rapidly up-regulated only on induction of monocytic differentiation. The rapidity of the responses may suggest that these factors play a regulatory role in lineage determination, where commitment to a particular differentiation pathway requires the loss/activation of these factors. However, as with all observations of this nature, whether these

changes are causal or a consequence of the differentiation program, remains to be tested.

(ii) Future prospects

Despite the problem of the defensin genes not being expressed in a model system, this research has opened up a number of potential avenues of fruitful research. This further work falls into two categories. The first is a continuation of the study of the regulation of this gene, and the second is the role of a number of the transcription factors (which have been implicated in the regulation of the defensin gene) in myeloid differentiation.

The first of these is a logical extension of the work presented here which leaves a number of questions unanswered or in need of further confirmation. Perhaps most obviously is the confirmation of the identity of the transcription factors which comprise the FP1, FP2 and FP3 activities. As a number of candidates have been implicated, this question could be addressed directly with antibodies employing super shift studies. As, however, the factors may be comprised of transcription factors which are novel, or ones against which no antibodies are available, the estimation of the molecular weights of the complexes would be informative. This could be undertaken by the use of biotinylated oligonucleotide capture with labelled nuclear extracts or UV. cross-linking of HL60 extracts to bromodeoxyuridine oligonucleotide probes.

The defensin genes are regulated in a differentiation-stage-specific manner, and the data in this study have allowed us to generate a model for this very specific transcriptional control. Clearly, the effect of differentiation along both pathways on the activity of the reporter constructs would be informative. This would allow us to test if the model holds in this cell line. This approach would require the generation of stable HL60 cell lines containing the reporter

constructs used in this work. The generation of stably transfected cell lines has been successfully undertaken in this laboratory using the neomycin resistance gene as a selectable marker (Kevin Ryan personal communication). A problem for these studies is that this marker affects the differentiation properties of this cell line (von-Melcher and Housman 1988), which is clearly not ideal for these studies. As an alternative the histidinol dehydrogenase gene was used a selectable marker which does not have this problem (Hartman and Mulligan 1988), but problems with the sensitivity of the cells varying with the density of the culture, and the generation of histidinol-resistant cells in mock transfected cultures, prevented use of this marker. Work currently underway (K.Ryan personal communication) suggests the use of the hygromycin resistance gene will provide a successful protocol for the generation of these lines.

The suggestion that the defensin genes are the homologues of the chicken *mim-1* gene also merits further study. Given the ability of C/EBP family members in conjunction with c-Myb to switch on expression of myeloid-specific genes in heterologous cell types when over-expressed, experiments to test if these transcription factors can act in a similar manner to induce expression of the defensin and lysozyme or other myeloid-specific genes would be interesting. Similarly, further work on the composite Myb-C/EBP element to test if these transcription factors can bind simultaneously would be interesting.

More generally with respect to C/EBP, this work has added weight to the suggested importance of these factors in myeloid differentiation. Clearly further work is merited, to determine if the C/EBP family is restricted to the myeloid compartment. Data from this work shows that the FP1 binding activities are lost very rapidly during induced granulocytic but not monocytic differentiation. This suggests the intriguing possibility that this factor(s) could be involved in lineage choice, between the monocytic and granulocytic lineages, perhaps with C/EBP activity in general playing an important role in committing progenitors to the

myeloid lineage, and individual C/EBP family members determining the granulocytic/monocytic choice. It will be important to determine if the results in the HL60 cell line can be repeated in other cell lines during differentiation, to assess if this pattern of FP1 binding activity is representative of that which takes place *in vivo* or is an artefact of this cell line. Given the suggested important role of this family of transcription factors in differentiation, it would be particularly interesting to express these transcription factors constitutively in the HL60 cell line to determine if they can change the differentiation properties of this cell line. To assess the effect of FP1 factor(s) it must be first determined which C/EBP members comprise this activity. Other interesting experiments using this approach could be the use of dominant negative mutants of C/EBP (Ness *et al.*, 1993; Ron and Habenauer 1992) in transfection studies. It is possible that negative regulators of C/EBP activity could play a role *in vivo* in the myeloid compartment as the rat C/EBPβ (LAP) has two translation start sites which generate a full length protein and a truncated protein LIP. This truncated protein can negatively regulate C/EBP activity at sub-stoichiometric amounts, and its level is regulated during hepatocyte differentiation (Descombes and Schibler 1991).

Part 5: References

Chapter 11: References

Allegra, P., I. R. Lonigro, F. Altieri, A. Tomassetti, A. Reale, and P. Caiafa. "Tightly bound non-histone proteins: distribution of tissue-specific components in the chromatin organization." Basic Appl Histochem 31 (3 1987): 247-53.

Almer, A. and W. Horz. "Nuclease hypersensitive regions with adjacent positioned nucleosomes mark the gene boundaries of the PHO5/PHO3 locus in yeast." Embo J 5 (10 1986): 2681-7.

Almer, A., H. Rudolph, A. Hinnen, and W. Horz. "Removal of positioned nucleosomes from the yeast PHO5 promoter upon PHO5 induction releases additional upstream activating DNA elements." Embo J 5 (10 1986): 2689-96.

Aman, P., D. Ron, N. Mandahl, T. Fioretos, S. Heim, K. Arheden, H. Willen, A. Rydholm, and F. Mitelman. "Rearrangement of the transcription factor gene CHOP in myxoid liposarcomas with t(12;16)(q13;p11)." Genes Chromosom Cancer 5 (4 1992): 278-85.

Amati, B., S. Dalton, M. W. Brooks, T. D. Littlewood, G. I. Evan, and H. Land. "Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max." Nature 359 (6394 1992): 423-6.

Ammendola, R., F. Gounari, G. Piaggio, V. De-Simone, and R. Cortese. "Transcription of the promoter of the rat NF-1 gene depends on the integrity of an Sp1 recognition site." Mol Cell Biol 10 (1 1990): 387-90.

Andersson, L. C., M. Jokinen, and C. G. Gahmberg. "Induction of erythroid differentiation in the human leukaemia cell line K562." Nature 278 (5702 1979): 364-5.

Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. "Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor." Cell 49 (6 1987): 729-39.

Annunziato, A. T., L. L. Frado, R. L. Seale, and C. L. Woodcock. "Treatment with sodium butyrate inhibits the complete condensation of interphase chromatin." Chromosoma 96 (2 1988): 132-8.

Antequera, F. and A. Bird. "Number of CpG islands and genes in human and mouse." Proc Natl Acad Sci U S A 90 (24 1993): 11995-9.

Antequera, F., J. Boyes, and A. Bird. "High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines." Cell 62 (3 1990): 503-14.

Archer, T. K., P. Lefebvre, R. G. Wolford, and G. L. Hager. "Transcription factor loading on the MMTV promoter: a bimodal mechanism for promoter activation [published erratum appears in Science 1992 Apr 10;256(5054):161]." Science 255 (5051 1992): 1573-6.

Arsura, M., M. Introna, F. Passerini, A. Mantovani, and J. Golay. "B-myb antisense oligonucleotides inhibit proliferation of human hematopoietic cell lines." Blood 79 (10 1992): 2708-16.

Askew, D. S., R. A. Ashmun, B. C. Simmons, and J. L. Cleveland. "Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis." Oncogene 6 (10 1991): 1915-22.

Ayer, D. E. and W. S. Dynan. "Simian virus 40 major late promoter: a novel tripartite structure that includes intragenic sequences." Mol Cell Biol 8 (5 1988): 2021-33.

Baer, R. "TAL1, TAL2 and LYL1: a family of basic helix-loop-helix proteins implicated in T cell acute leukaemia." Semin Cancer Biol 4 (6 1993): 341-7.

Bakhshi, A., J. P. Jensen, P. Goldman, J. J. Wright, O. W. McBride, A. L. Epstein, and S. J. Korsmeyer. "Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18." Cell 41 (3 1985): 899-906.

Bakker, O. and M. G. Parker. "CAAT/enhancer binding protein is able to bind to ATF/CRE elements." Nucleic Acids Res 19 (6 1991): 1213-7.

Ball, D. J., D. S. Gross, and W. T. Garrard. "5-methylcytosine is localized in nucleosomes that contain histone H1." Proc Natl Acad Sci U S A 80 (18 1983): 5490-4.

Band, H., V. Corredor, J. Lustgarten, K. Huebner, J. D. Griffin, D. W. Kufe, and E. J. Yunis. "Southern blot analysis of BII cell line--a putative variant of HL-60." Leukemia 1 (2 1987): 142-5.

Barry, M. A., J. E. Reynolds, and A. Eastman. "Etoposide-induced apoptosis in human HL-60 cells is associated with intracellular acidification." Cancer Res 53 (10 Suppl 1993): 2349-57.

Bartlett, J. D., J. D. Luethy, S. G. Carlson, S. J. Sollott, and N. J. Holbrook. "Calcium ionophore A23187 induces expression of the growth arrest and DNA damage inducible CCAAT/enhancer-binding protein (C/EBP)-related gene, gadd153. Ca2+ increases transcriptional activity and mRNA stability." J Biol Chem 267 (28 1992): 20465-70.

Baumhueter, S., G. Courtois, and G. R. Crabtree. "A variant nuclear protein in dedifferentiated hepatoma cells binds to the same functional sequences in the beta fibrinogen gene promoter as HNF-1." Embo J 7 (8 1988): 2485-93.

Bear, S. E., A. Bellacosa, P. A. Lazo, N. A. Jenkins, N. G. Copeland, C. Hanson, G. Levan, and P. N. Tsichlis. "Provirus insertion in Tpl-1, an Ets-1-related oncogene, is associated with tumor progression in Moloney murine leukemia virus-induced rat thymic lymphomas." Proc Natl Acad Sci U S A 86 (19 1989): 7495-9.

Bellen, H. J., C. J. O'Kane, C. Wilson, U. Grossniklaus, R. K. Pearson, and W. J. Gehring. "P-element-mediated enhancer detection: a versatile method to study development in Drosophila." Genes Dev 3 (9 1989): 1288-300.

Benbrook, D. M. and N. C. Jones. "Heterodimer formation between CREB and JUN proteins." Oncogene 5 (3 1990): 295-302.

Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub. "The protein Id: a negative regulator of helix-loop-helix DNA binding proteins." Cell 61 (1 1990): 49-59.

Bengal, E., L. Ransone, R. Scharfmann, V. J. Dwarki, S. J. Tapscott, H. Weintraub, and I. M. Verma. "Functional antagonism between c-Jun and MyoD proteins: a direct physical association." Cell 68 (3 1992): 507-19.

fection and disease progression." Anim Genet 20 (3 1989): 337-9.

Bhat, N. K., R. J. Fisher, S. Fujiwara, R. Ascione, and T. S. Papas. "Temporal and tissue-specific expression of mouse ets genes." Proc Natl Acad Sci U S A 84 (10 1987): 3161-5.

Bhat, N. K., C. B. Thompson, T. Lindsten, C. H. June, S. Fujiwara, S. Koizumi, R. J. Fisher, and T. S. Papas. "Reciprocal expression of human ETS1 and ETS2 genes during

T-cell activation: regulatory role for the protooncogene ETS1." Proc Natl Acad Sci U S A 87 (10 1990): 3723-7.

Bird, A., M. Taggart, M. Frommer, O. J. Miller, and D. Macleod. "A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA." Cell 40 (1 1985): 91-9.

Birkenmeier, E. H., B. Gwynn, S. Howard, J. Jerry, J. I. Gordon, W. H. Landschulz, and S. L. McKnight. "Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein." Genes Dev 3 (8 1989): 1146-56.

Blacklock, H. A., M. J. Gilmore, H. G. Prentice, G. R. Hazlehurst, J. P. Evans, D. D. Ma, C. B. Knight, and A. V. Hoffbrand. "ABO-incompatible bone-marrow transplantation: removal of red blood cells from donor marrow avoiding recipient antibody depletion." Lancet 2 (8307 1982): 1061-4.

Blackwood, E. M. and R. N. Eisenman. "Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc." Science 251 (4998 1991): 1211-7.

Bollum, F. J. "Development of antibodies and immunological methods for terminal transferase assay." Adv Exp Med Biol 145 (1982): 101-8.

Bonifer, C., M. Vidal, F. Grosveld, and A. E. Sippel. "Tissue specific and position independent expression of the complete gene domain for chicken lysozyme in transgenic mice." Embo J 9 (9 1990): 2843-8.

Borzillo, G. V., R. A. Ashmun, and C. J. Sherr. "Macrophage lineage switching of murine early pre-B lymphoid cells expressing transduced fms genes." Mol Cell Biol 10 (6 1990): 2703-14.

Bos, J. L. "ras oncogenes in human cancer: a review [published erratum appears in Cancer Res 1990 Feb 15;50(4):1352]." Cancer Res 49 (17 1989): 4682-9.

Bos, J. L., D. Toksoz, C. J. Marshall, M. Verlaan-de-Vries, G. H. Veeneman, A. J. van-der-Eb, J. H. van-Boom, J. W. Janssen, and A. C. Steenvoorden. "Amino-acid substitutions at codon 13 of the N-ras oncogene in human acute myeloid leukaemia." Nature 315 (6022 1985): 726-30.

Bos, J. L., M. Verlaan-de-Vries, A. M. Jansen, G. H. Veeneman, J. H. van-Boom, and A. J. van-der-Eb. "Three different mutations in codon 61 of the human N-ras gene detected by synthetic oligonucleotide hybridization." Nucleic Acids Res 12 (23 1984): 9155-63.

Boyes, J. and A. Bird. "DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein." Cell 64 (6 1991): 1123-34.

Boyes, J. and A. Bird. "Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein." Embo J 11 (1 1992): 327-33.

Bradstock, K. F., G. Pizzolo, E. S. Papageorgiou, G. L. Cetto, H. G. Prentice, K. Ganeshaguru, A. G. Prentice, A. Smith, M. Chilosi, A. Ambrosetti, and A. V. Hoffbrand. "Terminal transferase expression in relapsed acute myeloid leukaemia." Br J Haematol 49 (4 1981a): 621-7.

Breitman, T. R., S. J. Collins, and B. R. Keene. "Terminal differentiation of human promyelocytic leukemic cells in primary culture in response to retinoic acid." Blood 57 (6 1981): 1000-4.

Brennan, J. K., C. N. Abboud, J. F. DiPersio, G. H. Barlow, and M. A. Lichtman. "Autostimulation of growth by human myelogenous leukemia cells (HL-60)." Blood 58 (4 1981): 803-12.

Brennan, T. J., D. G. Edmondson, L. Li, and E. N. Olson. "Transforming growth factor beta represses the actions of myogenin through a mechanism independent of DNA binding." Proc Natl Acad Sci U S A 88 (9 1991): 3822-6.

Brent, R. and M. Ptashne. "A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor." Cell 43 (3 Pt 2 1985): 729-36.

Bresnick, E. H., M. Bustin, V. Marsaud, H. Richard-Foy, and G. L. Hager. "The transcriptionally-active MMTV promoter is depleted of histone H1." Nucleic Acids Res 20 (2 1992): 273-8.

Buck, M., H. Turler, and M. Chojkier. "LAP (NF-IL-6), a tissue-specific transcriptional activator, is an inhibitor of hepatoma cell proliferation." Embo J 13 (4 1994): 851-60.

Buratowski, S., S. Hahn, L. Guarente, and P. A. Sharp. "Five intermediate complexes in transcription initiation by RNA polymerase II." Cell 56 (4 1989): 549-61.

Burk, O., S. Mink, M. Ringwald, and K. H. Klempnauer. "Synergistic activation of the chicken mim-1 gene by v-myb and C/EBP transcription factors." Embo J 12 (5 1993): 2027-38.

Calabretta, B., R. B. Sims, M. Valtieri, D. Caracciolo, C. Szczylik, D. Venturelli, M. Ratajczak, M. Beran, and A. M. Gewirtz. "Normal and leukemic hematopoietic cells manifest differential sensitivity to inhibitory effects of c-myb antisense oligodeoxynucleotides: an in vitro study relevant to bone marrow purging." Proc Natl Acad Sci U S A 88 (6 1991): 2351-5.

Campo, M. S., D. A. Spandidos, J. Lang, and N. M. Wilkie. "Transcriptional control signals in the genome of bovine papillomavirus type 1." Nature 303 (5912 1983): 77-80.

Cancela, L., I. Nemere, and A. W. Norman. "1 alpha,25(OH)2 vitamin D3: a steroid hormone capable of producing pleiotropic receptor-mediated biological responses by both genomic and nongenomic mechanisms." J Steroid Biochem 30 (1-6 1988): 33-9.

Cao, Z., R. M. Umek, and S. L. McKnight. "Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells." Genes Dev 5 (9 1991): 1538-52.

Carcamo, J., E. Maldonado, P. Cortes, M. H. Ahn, I. Ha, Y. Kasai, J. Flint, and D. Reinberg. "A TATA-like sequence located downstream of the transcription initiation site is required for expression of an RNA polymerase II transcribed gene." Genes Dev 4 (9 1990): 1611-22.

Carey, M., Y. S. Lin, M. R. Green, and M. Ptashne. "A mechanism for synergistic activation of a mammalian gene by GAL4 derivatives." Nature 345 (6273 1990): 361-4.

Cereghini, S., M. Raymondjean, A. G. Carranca, P. Herbomel, and M. Yaniv. "Factors involved in control of tissue-specific expression of albumin gene." Cell 50 (4 1987): 627-38.

Chan, L. C., Y. L. Kwong, and S. Y. Ha. "Neurofibromatosis and increased risk of leukaemia--is the G-CSF gene involved?" Leukemia 5 (12 1991): 1113-4.

Chang, C. J., T. T. Chen, H. Y. Lei, D. S. Chen, and S. C. Lee. "Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family." Mol Cell Biol 10 (12 1990): 6642-53.

Chang, J. M., D. Metcalf, T. J. Gonda, and G. R. Johnson. "Long-term exposure to retrovirally expressed granulocyte-colony-stimulating factor induces a nonneoplastic granulocytic and progenitor cell hyperplasia without tissue damage in mice." J Clin Invest 84 (5 1989): 1488-96.

Charp, P. A., W. G. Rice, R. L. Raynor, E. Reimund, Jm Kinkade Jr., T. Ganz, M. E. Selsted, R. I. Lehrer, and J. F. Kuo. "Inhibition of protein kinase C by defensins, antibiotic peptides from human neutrophils." Biochem Pharmacol 37 (5 1988): 951-6.

Chasman, D. I., J. Leatherwood, M. Carey, M. Ptashne, and R. D. Kornberg. "Activation of yeast polymerase II transcription by herpesvirus VP16 and GAL4 derivatives in vitro." Mol Cell Biol 9 (11 1989): 4746-9.

Chen, H. M., H. L. Pahl, R. J. Scheibe, D. E. Zhang, and D. G. Tenen. "The Sp1 transcription factor binds the CD11b promoter specifically in myeloid cells in vivo and is essential for myeloid-specific promoter activity." J Biol Chem 268 (11 1993): 8230-9.

Cherry, J. R., T. R. Johnson, C. Dollard, J. R. Shuster, and C. L. Denis. "Cyclic AMP-dependent protein kinase phosphorylates and inactivates the yeast transcriptional activator ADR1." Cell 56 (3 1989): 409-19.

Choi, Y. C. and C. B. Chae. "Demethylation of somatic and testis-specific histone H2A and H2B genes in F9 embryonal carcinoma cells." Mol Cell Biol 13 (9 1993): 5538-48.

Chomienne, C., M. Cornic, S. Castaigne, P. Lefebvre, H. de-The, A. Dejean, and L. Degos. "[Biological parameters of the efficiency of retinoic acid in acute leukemia]." C R Seances Soc Biol Fil 185 (6 1991): 456-63.

Christy, R. J., K. H. Kaestner, D. E. Geiman, and M. D. Lane. "CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes." Proc Natl Acad Sci U S A 88 (6 1991): 2593-7.

Christy, R. J., V. W. Yang, J. M. Ntambi, D. E. Geiman, W. H. Landschulz, A. D. Friedman, Y. Nakabeppu, T. J. Kelly, and M. D. Lane. "Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes." Genes Dev 3 (9 1989): 1323-35.

Clarke, M. F., J. F. Kukowska-Latallo, E. Westin, M. Smith, and E. V. Prochownik. "Constitutive expression of a c-myb cDNA blocks Friend murine erythroleukemia cell differentiation." Mol Cell Biol 8 (2 1988): 884-92.

Cleary, M. L. and J. Sklar. "Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18." Proc Natl Acad Sci U S A 82 (21 1985): 7439-43.

Clerc, R. G., L. M. Corcoran, J. H. LeBowitz, D. Baltimore, and P. A. Sharp. "The B-cell-specific Oct-2 protein contains POU box- and homeo box-type domains." Genes Dev 2 (12a 1988): 1570-81.

Coebergh, J. W., A. van-der-Does-van-den-Berg, E. R. van-Wering, H. A. van-Steensel-Moll, H. A. Valkenburg, M. B. van't-Veer, P. I. Schmitz, and G. E. van-Zanen. "Childhood leukaemia in The Netherlands, 1973-1986: temporary variation of the incidence of acute lymphocytic leukaemia in young children." Br J Cancer 59 (1 1989): 100-5.

Coebergh, J. W., H. A. van-Steensel-Moll, E. R. Van-Wering, and M. B. van't-Veer. "Epidemiological and immunological characteristics of childhood leukaemia in The Netherlands: population-based data from a nationwide co-operative group of paediatricians." Leuk Res 9 (6 1985): 683-8.

Cohen, D. R. and T. Curran. "Analysis of dimerization and DNA binding functions in Fos and Jun by domain-swapping: involvement of residues outside the leucine zipper/basic region." Oncogene 5 (6 1990): 929-39.

Coleman, M. S. and J. J. Hutton. "Use of immunofluorescence tests for TdT in human hematologic malignancy." Adv Exp Med Biol 145 (1982): 109-14.

Collins, S. and M. Groudine. "Amplification of endogenous myc-related DNA sequences in a human myeloid leukaemia cell line." Nature 298 (5875 1982): 679-81.

Collins, S. J. "The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression." Blood 70 (5 1987): 1233-44.

Collins, S. J., F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo. "Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds." Proc Natl Acad Sci U S A 75 (5 1978a): 2458-62.

Collins, S. J., F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo. "Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds." Proc Natl Acad Sci U S A 75 (5 1978b): 2458-62.

Craig, R. W., O. S. Frankfurt, H. Sakagami, K. Takeda, and A. Bloch. "Macromolecular and cell cycle effects of different classes of agents inducing the maturation of human myeloblastic leukemia (ML-1) cells." Cancer Res 44 (6 1984): 2421-9.

Cross, M. and R. Renkawitz. "Repetitive sequence involvement in the duplication and divergence of mouse lysozyme genes." Embo J 9 (4 1990): 1283-8.

Daher, K. A., R. I. Lehrer, T. Ganz, and M. Kronenberg. "Isolation and characterization of human defensin cDNA clones [published erratum appears in Proc Natl Acad Sci U S A 1989 Jan;86(1):342]." Proc Natl Acad Sci U S A 85 (19 1988): 7327-31.

Daher, K. A., M. E. Selsted, and R. I. Lehrer. "Direct inactivation of viruses by human granulocyte defensins." J Virol 60 (3 1986): 1068-74.

Dalton, S. and R. Treisman. "Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element." Cell 68 (3 1992): 597-612.

Damm, K., R. A. Heyman, K. Umesono, and R. M. Evans. "Functional inhibition of retinoic acid response by dominant negative retinoic acid receptor mutants." Proc Natl Acad Sci U S A 90 (7 1993): 2989-93.

de-The, H., C. Lavau, A. Marchio, C. Chomienne, L. Degos, and A. Dejean. "The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR." Cell 66 (4 1991): 675-84.

Delattre, O., J. Zucman, B. Plougastel, C. Desmaze, T. Melot, M. Peter, H. Kovar, I. Joubert, P. de-Jong, G. Rouleau, and al et. "Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours." Nature 359 (6391 1992): 162-5.

Descombes, P., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler. "LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein." Genes Dev 4 (9 1990): 1541-51.

Descombes, P. and U. Schibler. "A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA." Cell 67 (3 1991): 569-79.

Dexter, T. M. "Stromal cell associated haemopoiesis." J Cell Physiol Suppl 1 (1982): 87-94.

Dexter, T. M. and E. Spooncer. "Growth and differentiation in the hemopoietic system." Annu Rev Cell Biol 3 (1987a): 423-41.

Donti, E., A. Tabilio, G. V. Donti, C. Mecucci, A. Carotti, F. Grignani, and M. F. Martelli. "5q- syndrome terminating in acute myeloid leukemia. Karyotype evolution and immunologic characterization of blast cells." Cancer Genet Cytogenet 32 (2 1988): 205-9.

Dorn, A., J. Bollekens, A. Staub, C. Benoist, and D. Mathis. "A multiplicity of CCAAT box-binding proteins." Cell 50 (6 1987): 863-72.

Dudek, H. and E. P. Reddy. "Murine myeloid leukemias with aberrant myb loci show heterogeneous expression of novel myb proteins." Oncogene 4 (12 1989): 1489-95.

Dudek, H., R. V. Tantravahi, V. N. Rao, E. S. Reddy, and E. P. Reddy. "Myb and Ets proteins cooperate in transcriptional activation of the mim-1 promoter." Proc Natl Acad Sci U S A 89 (4 1992): 1291-5.

Duncan, M., E. M. DiCicco-Bloom, X. Xiang, R. Benezra, and K. Chada. "The gene for the helix-loop-helix protein, Id, is specifically expressed in neural precursors." Dev Biol 154 (1 1992): 1-10.

Durrin, L. K., R. K. Mann, and M. Grunstein. "Nucleosome loss activates CUP1 and HIS3 promoters to fully induced levels in the yeast Saccharomyces cerevisiae." Mol Cell Biol 12 (4 1992): 1621-9.

Durrin, L. K., R. K. Mann, P. S. Kayne, and M. Grunstein. "Yeast histone H4 N-terminal sequence is required for promoter activation in vivo." Cell 65 (6 1991): 1023-31.

Dvorak, M., P. Urbanek, P. Bartunek, V. Paces, J. Vlach, V. Pecenka, L. Arnold, M. Travnicek, and J. Riman. "Transcription of the chicken myb proto-oncogene starts within a CpG island." Nucleic Acids Res 17 (14 1989): 5651-64.

el-Deiry, W. S., J. W. Harper, P. M. O'Connor, V. E. Velculescu, C. E. Canman, J. Jackman, J. A. Pietenpol, M. Burrell, D. E. Hill, Y. Wang, and al et. "WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis." Cancer Res 54 (5 1994): 1169-74.

Evans, J. L., T. L. Moore, W. M. Kuehl, T. Bender, and J. P. Ting. "Functional analysis of c-Myb protein in T-lymphocytic cell lines shows that it trans-activates the c-myc promoter." Mol Cell Biol 10 (11 1990): 5747-52.

Fairbairn, L. J., G. J. Cowling, B. M. Reipert, and T. M. Dexter. "Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors." Cell 74 (5 1993): 823-32.

Falkner, F. G. and H. G. Zachau. "Correct transcription of an immunoglobulin kappa gene requires an upstream fragment containing conserved sequence elements." Nature 310 (5972 1984): 71-4.

Fanidi, A., E. A. Harrington, and G. I. Evan. "Cooperative interaction between c-myc and bcl-2 proto-oncogenes." Nature 359 (6395 1992): 554-6.

Fascher, K. D., J. Schmitz, and W. Horz. "Role of trans-activating proteins in the generation of active chromatin at the PHO5 promoter in S. cerevisiae." Embo J 9 (8 1990): 2523-8.

Ferrari, S., A. Donelli, R. Manfredini, M. Sarti, R. Roncaglia, E. Tagliafico, E. Rossi, G. Torelli, and U. Torelli. "Differential effects of c-myb and c-fes antisense oligodeoxynucleotides on granulocytic differentiation of human myeloid leukemia HL60 cells." Cell Growth Differ 1 (11 1990): 543-8.

Fisher, R. J., G. Mavrothalassitis, A. Kondoh, and T. S. Papas. "High-affinity DNA-protein interactions of the cellular ETS1 protein: the determination of the ETS binding motif." Oncogene 6 (12 1991): 2249-54.

Fletcher, C., N. Heintz, and R. G. Roeder. "Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene." Cell 51 (5 1987): 773-81.

Foon, K. A. and Rf 3d Todd. "Immunologic classification of leukemia and lymphoma." Blood 68 (1 1986): 1-31.

Foos, G., S. Grimm, and K. H. Klempnauer. "Functional antagonism between members of the myb family: B-myb inhibits v-myb-induced gene activation." Embo J 11 (12 1992): 4619-29.

Frampton, J., T. J. Gibson, S. A. Ness, G. Doderlein, and T. Graf. "Proposed structure for the DNA-binding domain of the Myb oncoprotein based on model building and mutational analysis." Protein Eng 4 (8 1991): 891-901.

Frampton, J., A. Leutz, T. Gibson, and T. Graf. "DNA-binding domain ancestry [letter]." Nature 342 (6246 1989): 134.

Freytag, S. O. and T. J. Geddes. "Reciprocal regulation of adipogenesis by Myc and C/EBP alpha." Science 256 (5055 1992): 379-82.

Friedman, A. D., W. H. Landschulz, and S. L. McKnight. "CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells." Genes Dev 3 (9 1989): 1314-22.

Fujiwara, S., R. J. Fisher, N. K. Bhat, S. M. Diaz-de-la-Espina, and T. S. Papas. "A short-lived nuclear phosphoprotein encoded by the human ets-2 proto-oncogene is stabilized by activation of protein kinase C." Mol Cell Biol 8 (11 1988): 4700-6.

Fujiwara, S., S. Koizumi, R. J. Fisher, N. K. Bhat, and T. S. Papas. "Phosphorylation of the ETS-2 protein: regulation by the T-cell antigen receptor-CD3 complex." Mol Cell Biol 10 (3 1990): 1249-53.

Fukuhara, S., J. D. Rowley, D. Variakojis, and H. M. Golomb. "Chromosome abnormalities in poorly differentiated lymphocytic lymphoma." Cancer Res 39 (8 1979a): 3119-28.

Fukuhara, S., Y. Ueshima, S. Shirakawa, H. Uchino, and S. Morikawa. "14q translocations, having a break point at 14q13, in lymphoid malignancy." Int J Cancer 24 (6 1979b): 739-43.

Furley, A. J., S. Mizutani, K. Weilbaecher, H. S. Dhaliwal, A. M. Ford, L. C. Chan, H. V. Molgaard, B. Toyonaga, T. Mak, P. van-den-Elsen, and al et. "Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex." Cell 46 (1 1986): 75-87.

Gabrielsen, O. S., A. Sentenac, and P. Fromageot. "Specific DNA binding by c-Myb: evidence for a double helix-turn-helix-related motif." Science 253 (5024 1991): 1140-3.

Gallagher, R., S. Collins, J. Trujillo, K. McCredie, M. Ahearn, S. Tsai, R. Metzgar, G. Aulakh, R. Ting, F. Ruscetti, and R. Gallo. "Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia." Blood 54 (3 1979): 713-33.

Galson, D. L., J. O. Hensold, T. R. Bishop, M. Schalling, A. D. D'Andrea, C. Jones, P. E. Auron, and D. E. Housman. "Mouse beta-globin DNA-binding protein B1 is identical to a

proto-oncogene, the transcription factor Spi-1/PU.1, and is restricted in expression to hematopoietic cells and the testis." Mol Cell Biol 13 (5 1993): 2929-41.

Galson, D. L. and D. E. Housman. "Detection of two tissue-specific DNA-binding proteins with affinity for sites in the mouse beta-globin intervening sequence 2." Mol Cell Biol 8 (1 1988): 381-92.

Ganser, A., G. Seipelt, A. Lindemann, O. G. Ottmann, S. Falk, M. Eder, F. Herrmann, R. Becher, K. Hoffken, T. Buchner, and al et. "Effects of recombinant human interleukin-3 in patients with myelodysplastic syndromes." Blood 76 (3 1990): 455-62.

Ganz, T., J. R. Rayner, E. V. Valore, A. Tumolo, K. Talmadge, and F. Fuller. "The structure of the rabbit macrophage defensin genes and their organ-specific expression." J Immunol 143 (4 1989): 1358-65.

Garrard, W. T. "Histone H1 and the conformation of transcriptionally active chromatin." Bioessays 13 (2 1991): 87-8.

Gasser, S. M. and U. K. Laemmli. "Improved methods for the isolation of individual and clustered mitotic chromosomes [published erratum appears in Exp Cell Res 1988 Apr;175(2):419]." Exp Cell Res 173 (1 1987): 85-98.

Gauthier-Rouviere, C., M. Basset, J. M. Blanchard, J. C. Cavadore, A. Fernandez, and N. J. Lamb. "Casein kinase II induces c-fos expression via the serum response element pathway and p67SRF phosphorylation in living fibroblasts." Embo J 10 (10 1991): 2921-30.

Ghosh, S. and D. Baltimore. "Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B." Nature 344 (6267 1990): 678-82.

Gille, H., A. D. Sharrocks, and P. E. Shaw. "Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter." Nature 358 (6385 1992): 414-7.

Gonzalez, G. A. and M. R. Montminy. "Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133." Cell 59 (4 1989): 675-80.

Gonzalez, G. A., K. K. Yamamoto, W. H. Fischer, D. Karr, P. Menzel, W. 3d Biggs, W. W. Vale, and M. R. Montminy. "A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence." Nature 337 (6209 1989): 749-52.

Gorski, K., M. Carneiro, and U. Schibler. "Tissue-specific in vitro transcription from the mouse albumin promoter." Cell 47 (5 1986): 767-76.

Graves, B. J., P. F. Johnson, and S. L. McKnight. "Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene." Cell 44 (4 1986): 565-76.

Greaves, M. F. ""Target" cells, cellular phenotypes, and lineage fidelity in human leukaemia." J Cell Physiol Suppl 1 (1982): 113-25.

Greaves, M. F., L. C. Chan, A. J. Furley, S. M. Watt, and H. V. Molgaard. "Lineage promiscuity in hemopoietic differentiation and leukemia." Blood 67 (1 1986): 1-11.

Greaves, M. F., D. Delia, J. Robinson, R. Sutherland, and R. Newman. "Exploitation of monoclonal antibodies: a "who's who" of haemopoietic malignancy." Blood Cells 7 (2 1981): 257-80.

Greaves, M. F., S. Mizutani, A. J. Furley, D. R. Sutherland, L. C. Chan, A. M. Ford, and H. V. Molgaard. "Differentiation-linked gene rearrangement and expression in acute lymphoblastic leukaemia." Clin Haematol 15 (3 1986): 621-39.

Greaves, W. L., W. A. Orenstein, A. R. Hinman, and W. S. Nersesian. "Clinical efficacy of rubella vaccine." Pediatr Infect Dis 2 (4 1983): 284-6.

Green, A. R. and C. G. Begley. "SCL and related hemopoietic helix-loop-helix transcription factors." Int J Cell Cloning 10 (5 1992): 269-76.

Green, H. and O. Kehinde. "An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion." Cell 5 (1 1975): 19-27.

Green, H. and O. Kehinde. "Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells." Cell 7 (1 1976): 105-13.

Greenwald, G. I. and T. Ganz. "Defensins mediate the microbicidal activity of human neutrophil granule extract against Acinetobacter calcoaceticus." Infect Immun 55 (6 1987): 1365-8.

Grewal, T., M. Theisen, U. Borgmeyer, T. Grussenmeyer, R. A. Rupp, A. Stief, F. Qian, A. Hecht, and A. E. Sippel. "The -6.1-kilobase chicken lysozyme enhancer is a multifactorial complex containing several cell-type-specific elements." Mol Cell Biol 12 (5 1992): 2339-50.

Grignani, F., P. F. Ferrucci, U. Testa, G. Talamo, M. Fagioli, M. Alcalay, A. Mencarelli, F. Grignani, C. Peschle, I. Nicoletti, and al et. "The acute promyelocytic leukemia-specific PML-RAR alpha fusion protein inhibits differentiation and promotes survival of myeloid precursor cells." Cell 74 (3 1993): 423-31.

Gross, D. S. and W. T. Garrard. "Nuclease hypersensitive sites in chromatin." Annu Rev Biochem 57 (1988): 159-97.

Grosveld, G. C., E. de-Boer, C. K. Shewmaker, and R. A. Flavell. "DNA sequences necessary for transcription of the rabbit beta-globin gene in vivo." Nature 295 (5845 1982): 120-6.

Grove, M. and M. Plumb. "C/EBP, NF-kappa B, and c-Ets family members and transcriptional regulation of the cell-specific and inducible macrophage inflammatory protein 1 alpha immediate-early gene." Mol Cell Biol 13 (9 1993): 5276-89.

Gu, W., J. W. Schneider, G. Condorelli, S. Kaushal, V. Mahdavi, and B. Nadal-Ginard. "Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation." Cell 72 (3 1993): 309-24.

Ha, K., N. Hozumi, A. Hrincu, and E. W. Gelfand. "Lineage specific classification of leukaemia: results of the analysis of sixty cases of childhood leukaemia." Br J Haematol 61 (2 1985): 237-49.

Haas, J. G., M. Strobel, A. Leutz, P. Wendelgass, C. Muller, E. Sterneck, G. Riethmuller, and H. W. Ziegler-Heitbrock. "Constitutive monocyte-restricted activity of NF-M, a nuclear factor that binds to a C/EBP motif." J Immunol 149 (1 1992): 237-43.

Hagiwara, M., A. Alberts, P. Brindle, J. Meinkoth, J. Feramisco, T. Deng, M. Karin, S. Shenolikar, and M. Montminy. "Transcriptional attenuation following cAMP induction requires PP-1-mediated dephosphorylation of CREB." Cell 70 (1 1992): 105-13.

Hagman, J. and R. Grosschedl. "An inhibitory carboxyl-terminal domain in Ets-1 and Ets-2 mediates differential binding of ETS family factors to promoter sequences of the mb-1 gene." Proc Natl Acad Sci U S A 89 (19 1992): 8889-93.

Ham, J. and M. G. Parker. "Regulation of gene expression by nuclear hormone receptors." Curr Opin Cell Biol 1 (3 1989): 503-11.

Han, M. and M. Grunstein. "Nucleosome loss activates yeast downstream promoters in vivo." Cell 55 (6 1988): 1137-45.

Han, S., A. Udvardy, and P. Schedl. "Chromatin structure of the 87A7 heat-shock locus during heat induction and recovery from heat shock." Biochim Biophys Acta 825 (2 1985): 154-60.

Hancock, W. W., W. A. Muller, and R. S. Cotran. "Interleukin 2 receptors are expressed by alveolar macrophages during pulmonary sarcoidosis and are inducible by lymphokine treatment of normal human lung macrophages, blood monocytes, and monocyte cell lines." J Immunol 138 (1 1987): 185-91.

Hara, J., K. Kawa-Ha, Y. Takihara, K. Yumura-Yagi, S. Ishihara, A. Tawa, T. W. Mak, S. Okada, and E. W. Gelfand. "Developmental process of the T-cell receptor alpha and delta gene assembly in B-cell precursor acute lymphoblastic leukaemia." Br J Haematol 78 (2 1991): 180-6.

Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge. "The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases." Cell 75 (4 1993): 805-16.

Harvey, R. P., A. J. Robins, and J. R. Wells. "Independently evolving chicken histone H2B genes: identification of a ubiquitous H2B-specific 5' element." Nucleic Acids Res 10 (23 1982a): 7851-63.

Harvey, R. P., A. J. Robins, and J. R. Wells. "Independently evolving chicken histone H2B genes: identification of a ubiquitous H2B-specific 5' element." Nucleic Acids Res 10 (23 1982b): 7851-63.

Hauser, H., T. Graf, H. Beug, I. Greiser-Wilke, W. Lindenmaier, M. Grez, H. Land, K. Giesecke, and G. Schutz. "Structure of the lysozyme gene and expression in the oviduct and macrophages." Hamatol Bluttransfus 26 (1981): 175-8.

Henthorn, P., P. Zervos, M. Raducha, H. Harris, and T. Kadesch. "Expression of a human placental alkaline phosphatase gene in transfected cells: use as a reporter for studies of gene expression." Proc Natl Acad Sci U S A 85 (17 1988): 6342-6.

Herbst, R. S., N. Friedman, Je Darnell Jr., and L. E. Babiss. "Positive and negative regulatory elements in the mouse albumin enhancer." Proc Natl Acad Sci U S A 86 (5 1989): 1553-7.

Herr, W., R. A. Sturm, R. G. Clerc, L. M. Corcoran, D. Baltimore, P. A. Sharp, H. A. Ingraham, M. G. Rosenfeld, M. Finney, G. Ruvkun, and al et. "The POU domain: a large conserved region in the mammalian pit-1, oct-1, oct-2, and Caenorhabditis elegans unc-86 gene products." Genes Dev 2 (12a 1988): 1513-6.

Higurashi, M. and R. D. Cole. "The combination of DNA methylation and H1 histone binding inhibits the action of a restriction nuclease on plasmid DNA." J Biol Chem 266 (13 1991): 8619-25.

Hill, C. P., J. Yee, M. E. Selsted, and D. Eisenberg. "Crystal structure of defensin HNP-3, an amphiphilic dimer: mechanisms of membrane permeabilization." Science 251 (5000 1991): 1481-5.

Hipskind, R. A., V. N. Rao, C. G. Mueller, E. S. Reddy, and A. Nordheim. "Ets-related protein Elk-1 is homologous to the c-fos regulatory factor p62TCF." Nature 354 (6354 1991): 531-4.

Ho, I. C., N. K. Bhat, L. R. Gottschalk, T. Lindsten, C. B. Thompson, T. S. Papas, and J. M. Leiden. "Sequence-specific binding of human Ets-1 to the T cell receptor alpha gene enhancer." Science 250 (4982 1990): 814-8.

Hoffbrand, A. V., K. Ganeshaguru, G. Janossy, M. F. Greaves, D. Catovsky, and R. K. Woodruff. "Terminal deoxynucleotidyl-transferase levels and membrane phenotypes in diagnosis of acute leukaemia." Lancet 2 (8037 1977a): 520-3.

Holt, J. T., R. L. Redner, and A. W. Nienhuis. "An oligomer complementary to c-myc mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation." Mol Cell Biol 8 (2 1988): 963-73.

Hongping, J., Lin, J., Su, Z., *et al.*, "induction of differebtiation in human promyelocytic HL-60 leukemia cells activates p21, Waf/CIP1, expression in the absence of p53." Oncogene 9, (1994): 3397-3406.

Hope, I. A., S. Mahadevan, and K. Struhl. "Structural and functional characterization of the short acidic transcriptional activation region of yeast GCN4 protein." Nature 333 (6174 1988): 635-40.

Hope, I. A. and K. Struhl. "Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast." Cell 46 (6 1986): 885-94.

Howe, K. M. and R. J. Watson. "Nucleotide preferences in sequence-specific recognition of DNA by c-myb protein." Nucleic Acids Res 19 (14 1991): 3913-9.

Hozumi, M. "Established leukemia cell lines: their role in the understanding and control of leukemia proliferation." Crit Rev Oncol Hematol 3 (3 1985): 235-77.

Huang, J. D., D. H. Schwyter, J. M. Shirokawa, and A. J. Courey. "The interplay between multiple enhancer and silencer elements defines the pattern of decapentaplegic expression." Genes Dev 7 (4 1993): 694-704.

Hunter, T. and M. Karin. "The regulation of transcription by phosphorylation." Cell 70 (3 1992): 375-87.

Ibanez, C. E. and J. S. Lipsick. "trans activation of gene expression by v-myb." Mol Cell Biol 10 (5 1990): 2285-93.

Ihle, J. N. and D. Askew. "Origins and properties of hematopoietic growth factor-dependent cell lines." Int J Cell Cloning 7 (2 1989): 68-91.

Imataka, H., K. Sogawa, K. Yasumoto, Y. Kikuchi, K. Sasano, A. Kobayashi, M. Hayami, and Y. Fujii-Kuriyama. "Two regulatory proteins that bind to the basic transcription element (BTE), a GC box sequence in the promoter region of the rat P-4501A1 gene." Embo J 11 (10 1992): 3663-71.

Imrie, K., M. A. Baker, H. A. Messner, and A. Keating. "Prolonged disease-free survival in an adult presenting with Burkitt-type acute lymphoblastic leukaemia and CNS disease." J R Soc Med 85 (1 1992): 47-8.

Ing, N. H., J. M. Beekman, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley. "Members of the steroid hormone receptor superfamily interact with TFIIB (S300-II)." J Biol Chem 267 (25 1992): 17617-23.

Introna, M., J. Golay, J. Frampton, T. Nakano, S. A. Ness, and T. Graf. "Mutations in v-myb alter the differentiation of myelomonocytic cells transformed by the oncogene." Cell 63 (6 1990): 1289-97.

Ishii, S., J. T. Kadonaga, R. Tjian, J. N. Brady, G. T. Merlino, and I. Pastan. "Binding of the Sp1 transcription factor by the human Harvey ras1 proto-oncogene promoter." Science 232 (4756 1986): 1410-3.

Ivashkiv, L. B., H. C. Liou, C. J. Kara, W. W. Lamph, I. M. Verma, and L. H. Glimcher. "mXBP/CRE-BP2 and c-Jun form a complex which binds to the cyclic AMP, but not to the 12-O-tetradecanoylphorbol-13-acetate, response element." Mol Cell Biol 10 (4 1990): 1609-21.

Jackson, D. A., K. E. Rowader, K. Stevens, C. Jiang, P. Milos, and K. S. Zaret. "Modulation of liver-specific transcription by interactions between hepatocyte nuclear factor 3 and nuclear factor 1 binding DNA in close apposition." Mol Cell Biol 13 (4 1993): 2401-10.

Jani, P., W. Verbi, M. F. Greaves, D. Bevan, and F. Bollum. "Terminal deoxynucleotidyl transferase in acute myeloid leukaemia." Leuk Res 7 (1 1983): 17-29.

Janknecht, R., R. A. Hipskind, T. Houthaeve, A. Nordheim, and H. G. Stunnenberg. "Identification of multiple SRF N-terminal phosphorylation sites affecting DNA binding properties." Embo J 11 (3 1992): 1045-54.

Jen, Y., H. Weintraub, and R. Benezra. "Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins." Genes Dev 6 (8 1992): 1466-79.

Johnson, G. R. "Effects of excess granulocyte-macrophage colony stimulating factor (GM-CSF) in mice infected with a GM-CSF retrovirus." Dev Biol Stand 69 (1988): 3-8.

Johnson, L. M., P. S. Kayne, E. S. Kahn, and M. Grunstein. "Genetic evidence for an interaction between SIR3 and histone H4 in the repression of the silent mating loci in Saccharomyces cerevisiae." Proc Natl Acad Sci U S A 87 (16 1990): 6286-90.

Johnson, P. F., W. H. Landschulz, B. J. Graves, and S. L. McKnight. "Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses." Genes Dev 1 (2 1987): 133-46.

Johnston, M. "Genetic evidence that zinc is an essential co-factor in the DNA binding domain of GAL4 protein." Nature 328 (6128 1987): 353-5.

Jones, K. A. and R. Tjian. "Sp1 binds to promoter sequences and activates herpes simplex virus 'immediate-early' gene transcription in vitro." Nature 317 (6033 1985): 179-82.

Jones, P. A., M. J. Wolkowicz, Wm 3d Rideout, F. A. Gonzales, C. M. Marziasz, G. A. Coetzee, and S. J. Tapscott. "De novo methylation of the MyoD1 CpG island during the establishment of immortal cell lines." Proc Natl Acad Sci U S A 87 (16 1990): 6117-21.

Jost, J. P., H. P. Saluz, and A. Pawlak. "Estradiol down regulates the binding activity of an avian vitellogenin gene repressor (MDBP-2) and triggers a gradual demethylation of the mCpG pair of its DNA binding site." Nucleic Acids Res 19 (20 1991): 5771-5.

Just, U., C. Stocking, E. Spooncer, T. M. Dexter, and W. Ostertag. "Expression of the GM-CSF gene after retroviral transfer in hematopoietic stem cell lines induces synchronous granulocyte-macrophage differentiation." Cell 64 (6 1991): 1163-73.

Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tjian. "Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain." Cell 51 (6 1987): 1079-90.

Kaestner, K. H., R. J. Christy, and M. D. Lane. "Mouse insulin-responsive glucose transporter gene: characterization of the gene and trans-activation by the CCAAT/enhancer binding protein." Proc Natl Acad Sci U S A 87 (1 1990): 251-5.

Kagan, B. L., M. E. Selsted, T. Ganz, and R. I. Lehrer. "Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes." Proc Natl Acad Sci U S A 87 (1 1990): 210-4.

Kakizuka, A., Wh Miller Jr., K. Umesono, Rp Warrell Jr., S. R. Frankel, V. V. Murty, E. Dmitrovsky, and R. M. Evans. "Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML." Cell 66 (4 1991): 663-74.

Kanei-Ishii, C., E. M. MacMillan, T. Nomura, A. Sarai, R. G. Ramsay, S. Aimoto, S. Ishii, and T. J. Gonda. "Transactivation and transformation by Myb are negatively regulated by a leucine-zipper structure." Proc Natl Acad Sci U S A 89 (7 1992): 3088-92.

Kanei-Ishii, C., A. Sarai, T. Sawazaki, H. Nakagoshi, D. N. He, K. Ogata, Y. Nishimura, and S. Ishii. "The tryptophan cluster: a hypothetical structure of the DNA-binding domain of the myb protooncogene product." J Biol Chem 265 (32 1990): 19990-5.

Kanei-Ishii, C., T. Yasukawa, R. I. Morimoto, and S. Ishii. "c-Myb-induced transactivation mediated by heat shock elements without sequence-specific DNA binding of c-Myb." J Biol Chem 269 (22 1994): 15768-75.

Kastner, P., A. Perez, Y. Lutz, C. Rochette-Egly, M. P. Gaub, and P. Chambon. "[Fusion proteins between PML and alpha-RAR in acute promyelocytic leukemia]." C R Seances Soc Biol Fil 185 (6 1991): 391-401.

Kastner, P., A. Perez, Y. Lutz, C. Rochette-Egly, M. P. Gaub, B. Durand, M. Lanotte, R. Berger, and P. Chambon. "Structure, localization and transcriptional properties of two classes of retinoic acid receptor alpha fusion proteins in acute promyelocytic leukemia (APL): structural similarities with a new family of oncoproteins." Embo J 11 (2 1992): 629-42.

Katz, S., E. Kowenz-Leutz, C. Muller, K. Meese, S. A. Ness, and A. Leutz. "The NF-M transcription factor is related to C/EBP beta and plays a role in signal transduction, differentiation and leukemogenesis of avian myelomonocytic cells." Embo J 12 (4 1993): 1321-32.

Kawaguchi, N., H. F. DeLuca, and M. Noda. "Id gene expression and its suppression by 1,25-dihydroxyvitamin D3 in rat osteoblastic osteosarcoma cells." Proc Natl Acad Sci U S A 89 (10 1992): 4569-72.

Kayne, P. S., U. J. Kim, M. Han, J. R. Mullen, F. Yoshizaki, and M. Grunstein. "Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast." Cell 55 (1 1988): 27-39.

Keegan, L., G. Gill, and M. Ptashne. "Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein." Science 231 (4739 1986): 699-704.

Kellum, R. and P. Schedl. "A position-effect assay for boundaries of higher order chromosomal domains." Cell 64 (5 1991): 941-50.

Kellum, R. and P. Schedl. "A group of scs elements function as domain boundaries in an enhancer-blocking assay." Mol Cell Biol 12 (5 1992): 2424-31.

Kim, S. H., J. C. Moores, D. David, J. G. Respess, D. J. Jolly, and T. Friedmann. "The organization of the human HPRT gene." Nucleic Acids Res 14 (7 1986): 3103-18.

Kim, S. J., R. Lafyatis, K. Y. Kim, P. Angel, H. Fujiki, M. Karin, M. B. Sporn, and A. B. Roberts. "Regulation of collagenase gene expression by okadaic acid, an inhibitor of protein phosphatases." Cell Regul 1 (3 1990): 269-78.

Klages, S., B. Mollers, and R. Renkawitz. "The involvement of demethylation in the myeloid-specific function of the mouse M lysozyme gene downstream enhancer." Nucleic Acids Res 20 (8 1992): 1925-32.

Klempnauer, K. H., H. Arnold, and H. Biedenkapp. "Activation of transcription by v-myb: evidence for two different mechanisms." Genes Dev 3 (10 1989): 1582-9.

Klempnauer, K. H., T. J. Gonda, and J. M. Bishop. "Nucleotide sequence of the retroviral leukemia gene v-myb and its cellular progenitor c-myb: the architecture of a transduced oncogene." Cell 31 (2 Pt 1 1982): 453-63.

Klinken, S. P., W. S. Alexander, and J. M. Adams. "Hemopoietic lineage switch: v-raf oncogene converts Emu-myc transgenic B cells into macrophages." Cell 53 (6 1988): 857-67.

Koeffler, H. P. "Human myelogenous leukemia: enhanced clonal proliferation in the presence of phorbol diesters." Blood 57 (2 1981): 256-60.

Koeffler, H. P., M. Bar-Eli, and M. C. Territo. "Phorbol ester effect on differentiation of human myeloid leukemia cell lines blocked at different stages of maturation." Cancer Res 41 (3 1981): 919-26.

Koeffler, H. P. and D. W. Golde. "Acute myelogenous leukemia: a human cell line responsive to colony-stimulating activity." Science 200 (4346 1978a): 1153-4.

Koeffler, H. P. and D. W. Golde. "Acute myelogenous leukemia: a human cell line responsive to colony-stimulating activity." Science 200 (4346 1978b): 1153-4.

Koeffler, H. P., C. Miller, M. A. Nicolson, J. Ranyard, and R. A. Bosselman. "Increased expression of p53 protein in human leukemia cells." Proc Natl Acad Sci U S A 83 (11 1986): 4035-9.

Koizumi, S., R. J. Fisher, S. Fujiwara, C. Jorcyk, N. K. Bhat, A. Seth, and T. S. Papas. "Isoforms of the human ets-1 protein: generation by alternative splicing and differential phosphorylation." Oncogene 5 (5 1990): 675-81.

Konig, E., K. Berthold, H. A. Hienz, and G. Brittinger. "Griseofulvin and chronic granulocytic leukaemia." Helv Med Acta 35 (2 1969): 103-7.

Korsmeyer, S. J., P. A. Hieter, J. V. Ravetch, D. G. Poplack, T. A. Waldmann, and P. Leder. "Developmental hierarchy of immunoglobulin gene rearrangements in human leukemic pre-B-cells." Proc Natl Acad Sci U S A 78 (11 1981): 7096-100.

Kouzarides, T. and E. Ziff. "Leucine zippers of fos, jun and GCN4 dictate dimerization specificity and thereby control DNA binding." Nature 340 (6234 1989): 568-71.

Kreider, B. L., R. Benezra, G. Rovera, and T. Kadesch. "Inhibition of myeloid differentiation by the helix-loop-helix protein Id." Science 255 (5052 1992): 1700-2.

Kruger, W. and I. Herskowitz. "A negative regulator of HO transcription, SIN1 (SPT2), is a nonspecific DNA-binding protein related to HMG1." Mol Cell Biol 11 (8 1991): 4135-46.

Kyriakis, J. M., H. App, X. F. Zhang, P. Banerjee, D. L. Brautigan, U. R. Rapp, and J. Avruch. "Raf-1 activates MAP kinase-kinase." Nature 358 (6385 1992): 417-21.

La-Bella, F., R. Zhong, and N. Heintz. "Cell type-specific expression of a human histone H1 gene." J Biol Chem 263 (5 1988): 2115-8.

Lamb, R.F. " Characterisation of a gene regulated during myeloid differentiation." PhD thesis. University of Glasgow (1990).

Lai, E. and Je Darnell Jr. "Transcriptional control in hepatocytes: a window on development." Trends Biochem Sci 16 (11 1991): 427-30.

Landschulz, W. H., P. F. Johnson, E. Y. Adashi, B. J. Graves, and S. L. McKnight. "Isolation of a recombinant copy of the gene encoding C/EBP." Genes Dev 2 (7 1988): 786-800.

Langer, S. J., D. M. Bortner, M. F. Roussel, C. J. Sherr, and M. C. Ostrowski. "Mitogenic signaling by colony-stimulating factor 1 and ras is suppressed by the ets-2 DNA-binding domain and restored by myc overexpression." Mol Cell Biol 12 (12 1992): 5355-62.

Lanham, G. R., F. J. Bollum, D. L. Williams, and S. A. Stass. "Simultaneous occurrence of terminal deoxynucleotidyl transferase and myeloperoxidase in individual leukemia blasts." Blood 64 (1 1984): 318-20.

Lassar, A. B., J. N. Buskin, D. Lockshon, R. L. Davis, S. Apone, S. D. Hauschka, and H. Weintraub. "MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer." Cell 58 (5 1989a): 823-31.

Lassar, A. B., M. J. Thayer, R. W. Overell, and H. Weintraub. "Transformation by activated ras or fos prevents myogenesis by inhibiting expression of MyoD1." Cell 58 (4 1989b): 659-67.

Laybourn, P. J. and J. T. Kadonaga. "Role of nucleosomal cores and histone H1 in regulation of transcription by RNA polymerase II." Science 254 (5029 1991): 238-45.

Lee, R. T., C. L. Peterson, A. F. Calman, I. Herskowitz, and J. J. O'Donnell. "Cloning of a human galactokinase gene (GK2) on chromosome 15 by complementation in yeast." Proc Natl Acad Sci U S A 89 (22 1992): 10887-91.

Lehrer, R. I., A. Barton, K. A. Daher, S. S. Harwig, T. Ganz, and M. E. Selsted. "Interaction of human defensins with Escherichia coli. Mechanism of bactericidal activity." J Clin Invest 84 (2 1989): 553-61.

Lehrer, R. I., D. Szklarek, T. Ganz, and M. E. Selsted. "Synergistic activity of rabbit granulocyte peptides against Candida albicans." Infect Immun 52 (3 1986): 902-4.

Lemarchandel, V., J. Ghysdael, V. Mignotte, C. Rahuel, and P. H. Romeo. "GATA and Ets cis-acting sequences mediate megakaryocyte-specific expression." Mol Cell Biol 13 (1 1993): 668-76.

Leprince, D., M. Duterque-Coquillaud, R. P. Li, C. Henry, A. Flourens, B. Debuire, and D. Stehelin. "Alternative splicing within the chicken c-ets-1 locus: implications for transduction within the E26 retrovirus of the c-ets proto-oncogene." J Virol 62 (9 1988): 3233-41.

Leprince, D., A. Gegonne, J. Coll, C. de-Taisne, A. Schneeberger, C. Lagrou, and D. Stehelin. "A putative second cell-derived oncogene of the avian leukaemia retrovirus E26." Nature 306 (5941 1983): 395-7.

Leutz, A., H. Beug, and T. Graf. "Purification and characterization of cMGF, a novel chicken myelomonocytic growth factor." Embo J 3 (13 1984): 3191-7.

Leutz, A., K. Damm, E. Sterneck, E. Kowenz, S. Ness, R. Frank, H. Gausepohl, Y. C. Pan, J. Smart, M. Hayman, and al et. "Molecular cloning of the chicken myelomonocytic growth factor (cMGF) reveals relationship to interleukin 6 and granulocyte colony stimulating factor." Embo J 8 (1 1989): 175-81.

Li, E., T. H. Bestor, and R. Jaenisch. "Targeted mutation of the DNA methyltransferase gene results in embryonic lethality." Cell 69 (6 1992): 915-26.

Liang, R., G. Cheng, M. S. Wat, S. Y. Ha, and L. C. Chan. "Childhood acute lymphoblastic leukaemia presenting with relapsing hypoplastic anaemia: progression of the same abnormal clone." Br J Haematol 83 (2 1993): 340-2.

Lim, F., N. Kraut, J. Framptom, and T. Graf. "DNA binding by c-Ets-1, but not v-Ets, is repressed by an intramolecular mechanism." Embo J 11 (2 1992): 643-52.

Lin, F. T. and M. D. Lane. "Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes." Genes Dev 6 (4 1992): 533-44.

Lin, Y. S., I. Ha, E. Maldonado, D. Reinberg, and M. R. Green. "Binding of general transcription factor TFIIB to an acidic activating region." Nature 353 (6344 1991): 569-71.

Lord, K. A., A. Abdollahi, B. Hoffman-Liebermann, and D. A. Liebermann. "Proto-oncogenes of the fos/jun family of transcription factors are positive regulators of myeloid differentiation." Mol Cell Biol 13 (2 1993): 841-51.

Lozzio, B. B., C. B. Lozzio, E. G. Bamberger, and A. S. Feliu. "A multipotential leukemia cell line (K-562) of human origin." Proc Soc Exp Biol Med 166 (4 1981): 546-50.

Lozzio, C. B. and B. B. Lozzio. "Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome." Blood 45 (3 1975): 321-34.

Luo, Y., H. Fujii, T. Gerster, and R. G. Roeder. "A novel B cell-derived coactivator potentiates the activation of immunoglobulin promoters by octamer-binding transcription factors." Cell 71 (2 1992): 231-41.

Luscher, B., E. Christenson, D. W. Litchfield, E. G. Krebs, and R. N. Eisenman. "Myb DNA binding inhibited by phosphorylation at a site deleted during oncogenic activation." Nature 344 (6266 1990): 517-22.

Macleod, K., D. Leprince, and D. Stehelin. "The ets gene family." Trends Biochem Sci 17 (7 1992): 251-6.

Mahoney, C. W., J. Shuman, S. L. McKnight, H. C. Chen, and K. P. Huang. "Phosphorylation of CCAAT-enhancer binding protein by protein kinase C attenuates site-selective DNA binding." J Biol Chem 267 (27 1992): 19396-403.

Maire, P., J. Wuarin, and U. Schibler. "The role of cis-acting promoter elements in tissue-specific albumin gene expression." Science 244 (4902 1989): 343-6.

Marie, J. P., C. A. Izaguirre, C. I. Civin, J. Mirro, and E. A. McCulloch. "The presence within single K-562 cells of erythropoietic and granulopoietic differentiation markers." Blood 58 (4 1981): 708-11.

Matthews, B. W., D. H. Ohlendorf, W. F. Anderson, and Y. Takeda. "Structure of the DNA-binding region of lac repressor inferred from its homology with cro repressor." Proc Natl Acad Sci U S A 79 (5 1982a): 1428-32.

Matthews, B. W., D. H. Ohlendorf, W. F. Anderson, and Y. Takeda. "Structure of the DNA-binding region of lac repressor inferred from its homology with cro repressor." Proc Natl Acad Sci U S A 79 (5 1982b): 1428-32.

McCulloch, E. A. "Lineage infidelity or lineage promiscuity?" Leukemia 1 (3 1987): 235.

McDonnell, T. J., N. Deane, F. M. Platt, G. Nunez, U. Jaeger, J. P. McKearn, and S. J. Korsmeyer. "bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation." Cell 57 (1 1989): 79-88.

McDonnell, T. J. and S. J. Korsmeyer. "Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18)." Nature 349 (6306 1991): 254-6.

McKnight, S. and R. Tjian. "Transcriptional selectivity of viral genes in mammalian cells." Cell 46 (6 1986): 795-805.

Means, A. L. and P. J. Farnham. "Transcription initiation from the dihydrofolate reductase promoter is positioned by HIP1 binding at the initiation site." Mol Cell Biol 10 (2 1990): 653-61.

Meeker, T. C., D. Hardy, C. Willman, T. Hogan, and J. Abrams. "Activation of the interleukin-3 gene by chromosome translocation in acute lymphocytic leukemia with eosinophilia [see comments]." Blood 76 (2 1990): 285-9.

Mendel, D. B., P. A. Khavari, P. B. Conley, M. K. Graves, L. P. Hansen, A. Admon, and G. R. Crabtree. "Characterization of a cofactor that regulates dimerization of a mammalian homeodomain protein." Science 254 (5039 1991): 1762-7.

Mercola, M., X. F. Wang, J. Olsen, and K. Calame. "Transcriptional enhancer elements in the mouse immunoglobulin heavy chain locus." Science 221 (4611 1983): 663-5.

Mermod, N., E. A. O'Neill, T. J. Kelly, and R. Tjian. "The proline-rich transcriptional activator of CTF/NF-I is distinct from the replication and DNA binding domain." Cell 58 (4 1989): 741-53.

Messner, H. A. and J. D. Griffin. "Biology of acute myeloid leukaemia." Clin Haematol 15 (3 1986): 641-67.

Metcalf, D. "Actions and interactions of G-CSF, LIF, and IL-6 on normal and leukemic murine cells." Leukemia 3 (5 1989): 349-55.

Metz, R. and E. Ziff. "cAMP stimulates the C/EBP-related transcription factor rNFIL-6 to trans-locate to the nucleus and induce c-fos transcription." Genes Dev 5 (10 1991): 1754-66.

Mihara, K., X. R. Cao, A. Yen, S. Chandler, B. Driscoll, A. L. Murphree, A. T'Ang, and Y. K. Fung. "Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product." Science 246 (4935 1989): 1300-3.

Miller, C. W., K. Young, D. Dumenil, B. P. Alter, J. M. Schofield, and A. Bank. "Specific globin mRNAs in human erythroleukemia (K562) cells." Blood 63 (1 1984): 195-200.

Minghetti, P. P. and A. W. Norman. "1,25(OH)2-vitamin D3 receptors: gene regulation and genetic circuitry." Faseb J 2 (15 1988): 3043-53.

Mirkovitch, J., M. E. Mirault, and U. K. Laemmli. "Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold." Cell 39 (1 1984): 223-32.

Miyaura, C., K. Onozaki, Y. Akiyama, T. Taniyama, T. Hirano, T. Kishimoto, and T. Suda. "Recombinant human interleukin 6 (B-cell stimulatory factor 2) is a potent inducer of differentiation of mouse myeloid leukemia cells (M1)." Febs Lett 234 (1 1988): 17-21.

Mizuguchi, G., H. Nakagoshi, T. Nagase, N. Nomura, T. Date, Y. Ueno, and S. Ishii. "DNA binding activity and transcriptional activator function of the human B-myb protein compared with c-MYB." J Biol Chem 265 (16 1990): 9280-4.

Moll, T., G. Tebb, U. Surana, H. Robitsch, and K. Nasmyth. "The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the S. cerevisiae transcription factor SWI5." Cell 66 (4 1991): 743-58.

Mollers, B., S. Klages, A. Wedel, M. Cross, E. Spooncer, T. M. Dexter, and R. Renkawitz. "The mouse M-lysozyme gene domain: identification of myeloid and differentiation specific DNasel hypersensitive sites and of a 3'-cis acting regulatory element." Nucleic Acids Res 20 (8 1992): 1917-24.

Mucenski, M. L., K. McLain, A. B. Kier, S. H. Swerdlow, C. M. Schreiner, T. A. Miller, D. W. Pietryga, Wj Scott Jr., and S. S. Potter. "A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis." Cell 65 (4 1991): 677-89.

Murray, E. J. and F. Grosveld. "Site specific demethylation in the promoter of human gamma-globin gene does not alleviate methylation mediated suppression." Embo J 6 (8 1987): 2329-35.

Murray, M. J., J. M. Cunningham, L. F. Parada, F. Dautry, P. Lebowitz, and R. A. Weinberg. "The HL-60 transforming sequence: a ras oncogene coexisting with altered myc genes in hematopoietic tumors." Cell 33 (3 1983): 749-57.

Murre, C., P. S. McCaw, and D. Baltimore. "A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins." Cell 56 (5 1989a): 777-83.

Murre, C., P. S. McCaw, H. Vaessin, M. Caudy, L. Y. Jan, Y. N. Jan, C. V. Cabrera, J. N. Buskin, S. D. Hauschka, A. B. Lassar, and al et. "Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence." Cell 58 (3 1989b): 537-44.

Nakajima, N., M. Horikoshi, and R. G. Roeder. "Factors involved in specific transcription by mammalian RNA polymerase II: purification, genetic specificity, and TATA box-promoter interactions of TFIID." Mol Cell Biol 8 (10 1988): 4028-40.

Nakano, T. and T. Graf. "Identification of genes differentially expressed in two types of v-myb-transformed avian myelomonocytic cells." Oncogene 7 (3 1992): 527-34.

Needleman, S. W., M. H. Kraus, S. K. Srivastava, P. H. Levine, and S. A. Aaronson. "High frequency of N-ras activation in acute myelogenous leukemia." Blood 67 (3 1986): 753-7.

Ness, S. A., H. Beug, and T. Graf. "v-myb dominance over v-myc in doubly transformed chick myelomonocytic cells." Cell 51 (1 1987): 41-50.

Ness, S. A., E. Kowenz-Leutz, T. Casini, T. Graf, and A. Leutz. "Myb and NF-M: combinatorial activators of myeloid genes in heterologous cell types." Genes Dev 7 (5 1993): 749-59.

Ness, S. A., A. Marknell, and T. Graf. "The v-myb oncogene product binds to and activates the promyelocyte-specific mim-1 gene." Cell 59 (6 1989): 1115-25.

Newburger, P. E., M. E. Chovaniec, J. S. Greenberger, and H. J. Cohen. "Functional changes in human leukemic cell line HL-60. A model for myeloid differentiation." J Cell Biol 82 (2 1979): 315-22.

Nguyen, H. Q., B. Hoffman-Liebermann, and D. A. Liebermann. "The zinc finger transcription factor Egr-1 is essential for and restricts differentiation along the macrophage lineage." Cell 72 (2 1993): 197-209.

Nibbs, R. J., K. Itoh, W. Ostertag, and P. R. Harrison. "Differentiation arrest and stromal cell-independent growth of murine erythroleukemia cells are associated with elevated

expression of ets-related genes but not with mutation of p53." Mol Cell Biol 13 (9 1993): 5582-92.

Nicolaides, N. C., R. Gualdi, C. Casadevall, L. Manzella, and B. Calabretta. "Positive autoregulation of c-myb expression via Myb binding sites in the 5' flanking region of the human c-myb gene." Mol Cell Biol 11 (12 1991): 6166-76.

Nishio, Y., H. Isshiki, T. Kishimoto, and S. Akira. "A nuclear factor for interleukin-6 expression (NF-IL6) and the glucocorticoid receptor synergistically activate transcription of the rat alpha 1-acid glycoprotein gene via direct protein-protein interaction." Mol Cell Biol 13 (3 1993): 1854-62.

Nomiyama, H., C. Fromental, J. H. Xiao, and P. Chambon. "Cell-specific activity of the constituent elements of the simian virus 40 enhancer." Proc Natl Acad Sci U S A 84 (22 1987): 7881-5.

Nomura, N., M. Takahashi, M. Matsui, S. Ishii, T. Date, S. Sasamoto, and R. Ishizaki. "Isolation of human cDNA clones of myb-related genes, A-myb and B-myb [published erratum appears in Nucleic Acids Res 1989 Feb 11;17(3):1282]." Nucleic Acids Res 16 (23 1988): 11075-89.

Nomura, T., N. Sakai, A. Sarai, T. Sudo, C. Kanei-Ishii, R. G. Ramsay, D. Favier, T. J. Gonda, and S. Ishii. "Negative autoregulation of c-Myb activity by homodimer formation through the leucine zipper." J Biol Chem 268 (29 1993): 21914-23.

Norman, C., M. Runswick, R. Pollock, and R. Treisman. "Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element." Cell 55 (6 1988): 989-1003.

Nunez, G., L. London, D. Hockenbery, M. Alexander, J. P. McKearn, and S. J. Korsmeyer. "Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines." J Immunol 144 (9 1990): 3602-10.

Nunn, M. F., P. H. Seeburg, C. Moscovici, and P. H. Duesberg. "Tripartite structure of the avian erythroblastosis virus E26 transforming gene." Nature 306 (5941 1983): 391-5.

Nyakatura, G., J. Jantschak, U. Notzel, S. Prosch, S. Rosenthal, and H. A. Rosenthal. "Molecular cloning of integrated proviral DNA of bovine leukaemia virus from virus-producing foetal lamb kidney cells." Folia Biol Praha 31 (2 1985): 115-20.

Nye, J. A. and B. J. Graves. "Alkylation interference identifies essential DNA contacts for sequence-specific binding of the eukaryotic transcription factor C/EBP." Proc Natl Acad Sci U S A 87 (10 1990): 3992-6.

O'Shea, E. K., R. Rutkowski, Wf 3d Stafford, and P. S. Kim. "Preferential heterodimer formation by isolated leucine zippers from fos and jun." Science 245 (4918 1989): 646-8.

O'Shea-Greenfield, A. and S. T. Smale. "Roles of TATA and initiator elements in determining the start site location and direction of RNA polymerase II transcription." J Biol Chem 267 (2 1992): 1391-402.

Ogawa, M., P. N. Porter, and T. Nakahata. "Renewal and commitment to differentiation of hemopoietic stem cells (an interpretive review)." Blood 61 (5 1983): 823-9.

Oka, T., A. Rairkar, and J. H. Chen. "Structural and functional analysis of the regulatory sequences of the ets-1 gene." Oncogene 6 (11 1991): 2077-83.

Old, L. J., C. Iritani, E. Stockert, E. A. Boyse, and H. A. Campbell. "Increased anti-leukaemia activity of E. coli asparaginase in mice infected with L.D.H.-elevating virus." Lancet 2 (569 1968): 684-5.

Olsson, I. and T. Olofsson. "Induction of differentiation in a human promyelocytic leukemic cell line (HL-60). Production of granule proteins." Exp Cell Res 131 (1 1981): 225-30.

Ondek, B., L. Gloss, and W. Herr. "The SV40 enhancer contains two distinct levels of organization." Nature 333 (6168 1988): 40-5.

Ondek, B., A. Shepard, and W. Herr. "Discrete elements within the SV40 enhancer region display different cell-specific enhancer activities." Embo J 6 (4 1987): 1017-25.

Ouellette, A. J., S. I. Miller, A. H. Henschen, and M. E. Selsted. "Purification and primary structure of murine cryptdin-1, a Paneth cell defensin." Febs Lett 304 (2-3 1992): 146-8.

Pahl, H. L., T. C. Burn, and D. G. Tenen. "Optimization of transient transfection into human myeloid cell lines using a luciferase reporter gene." Exp Hematol 19 (10 1991): 1038-41.

Pahl, H. L., A. G. Rosmarin, and D. G. Tenen. "Characterization of the myeloid-specific CD11b promoter." Blood 79 (4 1992): 865-70.

Pahl, H. L., R. J. Scheibe, D. E. Zhang, H. M. Chen, D. L. Galson, R. A. Maki, and D. G. Tenen. "The proto-oncogene PU.1 regulates expression of the myeloid-specific CD11b promoter." J Biol Chem 268 (7 1993): 5014-20.

Pandolfi, P. P., F. Grignani, M. Alcalay, A. Mencarelli, A. Biondi, F. LoCoco, F. Grignani, and P. G. Pelicci. "Structure and origin of the acute promyelocytic leukemia myl/RAR alpha cDNA and characterization of its retinoid-binding and transactivation properties." Oncogene 6 (7 1991): 1285-92.

Papas, T. S., D. K. Watson, N. Sacchi, S. Fujiwara, A. K. Seth, R. J. Fisher, N. K. Bhat, G. Mavrothalassitis, S. Koizumi, C. L. Jorcyk, and al et. "ETS family of genes in leukemia and Down syndrome." Am J Med Genet Suppl 7 (1990): 251-61.

Pardi, A., X. L. Zhang, M. E. Selsted, J. J. Skalicky, and P. F. Yip. "NMR studies of defensin antimicrobial peptides. 2. Three-dimensional structures of rabbit NP-2 and human HNP-1." Biochemistry 31 (46 1992): 11357-64.

Pascal, E. and R. Tjian. "Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism." Genes Dev 5 (9 1991): 1646-56.

Patterson, J., H. G. Prentice, M. K. Brenner, M. Gilmore, G. Janossy, K. Ivory, D. Skeggs, H. Morgan, J. Lord, H. A. Blacklock, and al et. "Graft rejection following HLA matched T-lymphocyte depleted bone marrow transplantation." Br J Haematol 63 (2 1986): 221-30.

Paz-Ares, J., D. Ghosal, U. Wienand, P. A. Peterson, and H. Saedler. "The regulatory c1 locus of Zea mays encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators." Embo J 6 (12 1987): 3553-8.

Pearson, A. D., H. A. Amineddine, M. Yule, S. Mills, D. R. Long, A. W. Craft, and J. M. Chessells. "The influence of serum methotrexate concentrations and drug dosage on outcome in childhood acute lymphoblastic leukaemia [see comments]." Br J Cancer 64 (1 1991): 169-73.

Pearson, A. D., S. Mills, H. A. Amineddine, D. R. Long, A. W. Craft, and J. M. Chessells. "Pharmacokinetics of oral and intramuscular methotrexate in children with acute lymphoblastic leukaemia." Cancer Chemother Pharmacol 20 (3 1987): 243-7.

Pedrazzoli, P., M. A. Bains, R. Watson, J. Fisher, T. G. Hoy, and A. Jacobs. "c-myc and c-myb oncoproteins during induced maturation of myeloid and erythroid human leukemic cell lines." Cancer Res 49 (24 Pt 1 1989): 6911-6.

Pei, D. Q. and C. H. Shih. "An "attenuator domain" is sandwiched by two distinct transactivation domains in the transcription factor C/EBP." Mol Cell Biol 11 (3 1991): 1480-7.

Perkins, S. L., P. E. Andreotti, S. K. Sinha, M. C. Wu, and A. A. Yunis. "Human myeloid leukemic cell (HL-60) autostimulator: relationship to colony-stimulating factor." Cancer Res 44 (11 1984): 5169-75.

Peters, C. W., A. E. Sippel, M. Vingron, and K. H. Klempnauer. "Drosophila and vertebrate myb proteins share two conserved regions, one of which functions as a DNA-binding domain." Embo J 6 (10 1987): 3085-90.

Peterson, C. L. and I. Herskowitz. "Characterization of the yeast SWI1, SWI2, and SWI3 genes, which encode a global activator of transcription." Cell 68 (3 1992): 573-83.

Peterson, C. L., W. Kruger, and I. Herskowitz. "A functional interaction between the C-terminal domain of RNA polymerase II and the negative regulator SIN1." Cell 64 (6 1991): 1135-43.

Pfeffer, U., N. Ferrari, F. Tosetti, and G. Vidali. "Histone acetylation in conjugating Tetrahymena thermophila [published erratum appears in J Cell Biol 1989 Dec;109(6 Pt 1):3214-7]." J Cell Biol 109 (3 1989): 1007-14.

Pierce, J. H., M. Ruggiero, T. P. Fleming, P. P. Di-Fiore, J. S. Greenberger, L. Varticovski, J. Schlessinger, G. Rovera, and S. A. Aaronson. "Signal transduction through the EGF receptor transfected in IL-3-dependent hematopoietic cells." Science 239 (4840 1988): 628-31.

Pinkert, C. A., D. M. Ornitz, R. L. Brinster, and R. D. Palmiter. "An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice." Genes Dev 1 (3 1987): 268-76.

Pognonec, P., K. E. Boulukos, and J. Ghysdael. "The c-ets-1 protein is chromatin associated and binds to DNA in vitro." Oncogene 4 (6 1989): 691-7.

Polansky, D. A., A. Yang, K. Brader, and D. M. Miller. "Control of lysozyme gene expression in differentiating HL-60 cells." Br J Haematol 60 (1 1985): 7-17.

Poli, V., F. P. Mancini, and R. Cortese. "IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP." Cell 63 (3 1990): 643-53.

Pongubala, J. M., S. Nagulapalli, M. J. Klemsz, S. R. McKercher, R. A. Maki, and M. L. Atchison. "PU.1 recruits a second nuclear factor to a site important for immunoglobulin kappa 3' enhancer activity." Mol Cell Biol 12 (1 1992): 368-78.

Prentice, H. G., H. A. Blacklock, G. Janossy, K. F. Bradstock, D. Skeggs, G. Goldstein, and A. V. Hoffbrand. "Use of anti-T-cell monoclonal antibody OKT3 to prevent acute graft-versus-host disease in allogeneic bone-marrow transplantation for acute leukaemia." Lancet 1 (8274 1982): 700-3.

Principato, M., J. L. Cleveland, U. R. Rapp, K. L. Holmes, J. H. Pierce, Hc 3d Morse, and S. P. Klinken. "Transformation of murine bone marrow cells with combined v-raf-v-myc oncogenes yields clonally related mature B cells and macrophages." Mol Cell Biol 10 (7 1990): 3562-8.

Prywes, R. and R. G. Roeder. "Inducible binding of a factor to the c-fos enhancer." Cell 47 (5 1986): 777-84.

Ptashne, M. "Gene regulation by proteins acting nearby and at a distance." Nature 322 (6081 1986): 697-701.

Pulverer, B. J., K. Hughes, C. C. Franklin, A. S. Kraft, S. J. Leevers, and J. R. Woodgett. "Co-purification of mitogen-activated protein kinases with phorbol ester-induced c-Jun kinase activity in U937 leukaemic cells." Oncogene 8 (2 1993): 407-15.

Qian, S., M. Capovilla, and V. Pirrotta. "The bx region enhancer, a distant cis-control element of the Drosophila Ubx gene and its regulation by hunchback and other segmentation genes." Embo J 10 (6 1991): 1415-25.

Rabbitts, T. H. and T. Boehm. "Structural and functional chimerism results from chromosomal translocation in lymphoid tumors." Adv Immunol 50 (1991): 119-46.

Ramsay, R. G., S. Ishii, Y. Nishina, G. Soe, and T. J. Gonda. "Characterization of alternate and truncated forms of murine c-myb proteins." Oncogene Res 4 (4 1989): 259-69.

Rao, V. N. and E. S. Reddy. "elk-1 domains responsible for autonomous DNA binding, SRE:SRF interaction and negative regulation of DNA binding." Oncogene 7 (11 1992): 2335-40.

Ray, B. K. and A. Ray. "Expression of the gene encoding alpha 1-acid glycoprotein in rabbit liver under acute-phase conditions involves induction and activation of beta and delta CCAAT-enhancer-binding proteins." Eur J Biochem 222 (3 1994): 891-900.

Ray, D., R. Bosselut, J. Ghysdael, M. G. Mattei, A. Tavitian, and F. Moreau-Gachelin. "Characterization of Spi-B, a transcription factor related to the putative oncoprotein Spi-1/PU.1." Mol Cell Biol 12 (10 1992): 4297-304.

Reik, A., G. Schutz, and A. F. Stewart. "Glucocorticoids are required for establishment and maintenance of an alteration in chromatin structure: induction leads to a reversible disruption of nucleosomes over an enhancer." Embo J 10 (9 1991): 2569-76.

Reisman, D. and V. Rotter. "Induced expression from the Moloney murine leukemia virus long terminal repeat during differentiation of human myeloid cells is mediated through its transcriptional enhancer." Mol Cell Biol 9 (8 1989a): 3571-5.

Richard-Foy, H. and G. L. Hager. "Sequence-specific positioning of nucleosomes over the steroid-inducible MMTV promoter." Embo J 6 (8 1987): 2321-8.

Ridge, S. A., M. Worwood, D. Oscier, A. Jacobs, and R. A. Padua. "FMS mutations in myelodysplastic, leukemic, and normal subjects." Proc Natl Acad Sci U S A 87 (4 1990): 1377-80.

Riggs, A. D. "X inactivation, differentiation, and DNA methylation." Cytogenet Cell Genet 14 (1 1975): 9-25.

Roman, C., J. S. Platero, J. Shuman, and K. Calame. "Ig/EBP-1: a ubiquitously expressed immunoglobulin enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP." Genes Dev 4 (8 1990): 1404-15.

Ron, D. and J. F. Habener. "CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription." Genes Dev 6 (3 1992): 439-53.

Roth, S. Y., M. Shimizu, L. Johnson, M. Grunstein, and R. T. Simpson. "Stable nucleosome positioning and complete repression by the yeast alpha 2 repressor are disrupted by amino-terminal mutations in histone H4." Genes Dev 6 (3 1992): 411-25.

Rouet, P., G. Raguenez, F. Tronche, M. Yaniv, C. N'Guyen, and J. P. Salier. "A potent enhancer made of clustered liver-specific elements in the transcription control sequences of human alpha 1-microglobulin/bikunin gene." J Biol Chem 267 (29 1992): 20765-73.

Rovigatti, U., J. Mirro, G. Kitchingman, G. Dahl, J. Ochs, S. Murphy, and S. Stass. "Heavy chain immunoglobulin gene rearrangement in acute nonlymphocytic leukemia." Blood 63 (5 1984): 1023-7.

Rutherford, T. R., J. B. Clegg, and D. J. Weatherall. "K562 human leukaemic cells synthesise embryonic haemoglobin in response to haemin." Nature 280 (5718 1979): 164-5.

Sachs, L. "Control of growth and normal differentiation in leukemic cells: regulation of the developmental program and restoration of the normal phenotype in myeloid leukemia." J Cell Physiol Suppl 1 (1982): 151-64.

Saikumar, P., R. Murali, and E. P. Reddy. "Role of tryptophan repeats and flanking amino acids in Myb-DNA interactions." Proc Natl Acad Sci U S A 87 (21 1990): 8452-6.

Saito, H., A. Bourinbaiar, M. Ginsburg, K. Minato, E. Ceresi, K. Yamada, D. Machover, J. Breard, and G. Mathe. "Establishment and characterization of a new human eosinophilic leukemia cell line." Blood 66 (6 1985): 1233-40.

Sakura, H., C. Kanei-Ishii, T. Nagase, H. Nakagoshi, T. J. Gonda, and S. Ishii. "Delineation of three functional domains of the transcriptional activator encoded by the c-myb protooncogene." Proc Natl Acad Sci U S A 86 (15 1989): 5758-62.

Sap, J., A. Munoz, K. Damm, Y. Goldberg, J. Ghysdael, A. Leutz, H. Beug, and B. Vennstrom. "The c-erb-A protein is a high-affinity receptor for thyroid hormone." Nature 324 (6098 1986): 635-40.

Sargent, M. G. and M. F. Bennett. "Identification in Xenopus of a structural homologue of the Drosophila gene snail." Development 109 (4 1990): 967-73.

Sawadogo, M. and R. G. Roeder. "Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region." Cell 43 (1 1985): 165-75.

Sawadogo, M. and A. Sentenac. "RNA polymerase B (II) and general transcription factors." Annu Rev Biochem 59 (1990): 711-54.

Schmidt, M. C., Q. Zhou, and A. J. Berk. "Sp1 activates transcription without enhancing DNA-binding activity of the TATA box factor." Mol Cell Biol 9 (8 1989): 3299-307.

Schneider, C., R. Sutherland, R. Newman, and M. Greaves. "Structural features of the cell surface receptor for transferrin that is recognized by the monoclonal antibody OKT9." J Biol Chem 257 (14 1982): 8516-22.

Schneikert, J., Y. Lutz, and B. Wasylyk. "Two independent activation domains in c-Ets-1 and c-Ets-2 located in non-conserved sequences of the ets gene family." Oncogene 7 (2 1992): 249-56.

Schorpp, M., W. Kugler, U. Wagner, and G. U. Ryffel. "Hepatocyte-specific promoter element HP1 of the Xenopus albumin gene interacts with transcriptional factors of mammalian hepatocytes." J Mol Biol 202 (2 1988): 307-20.

Schule, R., P. Rangarajan, S. Kliewer, L. J. Ransone, J. Bolado, N. Yang, I. M. Verma, and R. M. Evans. "Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor." Cell 62 (6 1990a): 1217-26.

Schule, R., K. Umesono, D. J. Mangelsdorf, J. Bolado, J. W. Pike, and R. M. Evans. "Jun-Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene." Cell 61 (3 1990b): 497-504.

Scott, L. M., C. I. Civin, P. Rorth, and A. D. Friedman. "A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells." Blood 80 (7 1992): 1725-35.

Scott, M. P. and A. J. Weiner. "Structural relationships among genes that control development: sequence homology between the Antennapedia, Ultrabithorax, and fushi tarazu loci of Drosophila." Proc Natl Acad Sci U S A 81 (13 1984): 4115-9.

Selden, R. F., K. B. Howie, M. E. Rowe, H. M. Goodman, and D. D. Moore. "Human growth hormone as a reporter gene in regulation studies employing transient gene expression." Mol Cell Biol 6 (9 1986): 3173-9.

Selsted, M. E. and S. S. Harwig. "Purification, primary structure, and antimicrobial activities of a guinea pig neutrophil defensin." Infect Immun 55 (9 1987): 2281-6.

Selsted, M. E. and S. S. Harwig. "Determination of the disulfide array in the human defensin HNP-2. A covalently cyclized peptide." J Biol Chem 264 (7 1989): 4003-7.

Selsted, M. E., Y. Q. Tang, W. L. Morris, P. A. McGuire, M. J. Novotny, W. Smith, A. H. Henschen, and J. S. Cullor. "Purification, primary structures, and antibacterial activities of beta-defensins, a new family of antimicrobial peptides from bovine neutrophils." J Biol Chem 268 (9 1993): 6641-8.

Sen, S., R. Takahashi, S. Rani, E. J. Freireich, and S. A. Stass. "Expression of differentially phosphorylated Rb and mutant p53 proteins in myeloid leukemia cell lines." Leuk Res 17 (8 1993): 639-47.

Seto, E., Y. Shi, and T. Shenk. "YY1 is an initiator sequence-binding protein that directs and activates transcription in vitro." Nature 354 (6350 1991): 241-5.

Shastry, B. S., S. Y. Ng, and R. G. Roeder. "Multiple factors involved in the transcription of class III genes in Xenopus laevis." J Biol Chem 257 (21 1982): 12979-86.

Shen-Ong, G. L., Rm Skurla Jr., J. D. Owens, and J. F. Mushinski. "Alternative splicing of RNAs transcribed from the human c-myb gene." Mol Cell Biol 10 (6 1990): 2715-22.

Sherr, C. J., C. W. Rettenmier, R. Sacca, M. F. Roussel, A. T. Look, and E. R. Stanley. "The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1." Cell 41 (3 1985): 665-76.

Shirakawa, F. and S. B. Mizel. "In vitro activation and nuclear translocation of NF-kappa B catalyzed by cyclic AMP-dependent protein kinase and protein kinase C." Mol Cell Biol 9 (6 1989): 2424-30.

Shrader, T. E. and D. M. Crothers. "Artificial nucleosome positioning sequences." Proc Natl Acad Sci U S A 86 (19 1989): 7418-22.

Simpson, V. J., T. E. Johnson, and R. F. Hammen. "Caenorhabditis elegans DNA does not contain 5-methylcytosine at any time during development or aging." Nucleic Acids Res 14 (16 1986): 6711-9.

Siu, G., M. Kronenberg, E. Strauss, R. Haars, T. W. Mak, and L. Hood. "The structure, rearrangement and expression of D beta gene segments of the murine T-cell antigen receptor." Nature 311 (5984 1984): 344-50.

Skinnider, L. F., T. Ha, S. Bergen, and H. C. Wang. "Characterisation of a new cell line (CJ18) from a patient with 'hairy' cell leukaemia." Scand J Haematol 35 (4 1985): 430-6.

Sorrentino, V., R. Pepperkok, R. L. Davis, W. Ansorge, and L. Philipson. "Cell proliferation inhibited by MyoD1 independently of myogenic differentiation." Nature 345 (6278 1990): 813-5.

Sparkes, R. S., M. Kronenberg, C. Heinzmann, K. A. Daher, I. Klisak, T. Ganz, and T. Mohandas. "Assignment of defensin gene(s) to human chromosome 8p23." Genomics 5 (2 1989): 240-4.

Spooncer, E., C. M. Heyworth, A. Dunn, and T. M. Dexter. "Self-renewal and differentiation of interleukin-3-dependent multipotent stem cells are modulated by stromal cells and serum factors." Differentiation 31 (2 1986): 111-8.

Stanfield, R. L., E. M. Westbrook, and M. E. Selsted. "Characterization of two crystal forms of human defensin neutrophil cationic peptide 1, a naturally occurring antimicrobial peptide of leukocytes." J Biol Chem 263 (12 1988): 5933-5.

Steinman, R. A, Hoffman, B, Iro, A, et al., "induction of p21 (WAF1/CIP1) during differentiation." Oncogene 9 (1994): 3389-3396.

Sterneck, E., C. Blattner, T. Graf, and A. Leutz. "Structure of the chicken myelomonocytic growth factor gene and specific activation of its promoter in avian myelomonocytic cells by protein kinases." Mol Cell Biol 12 (4 1992): 1728-35.

Stief, A., D. M. Winter, W. H. Stratling, and A. E. Sippel. "A nuclear DNA attachment element mediates elevated and position-independent gene activity." Nature 341 (6240 1989): 343-5.

Stone, J., T. de-Lange, G. Ramsay, E. Jakobovits, J. M. Bishop, H. Varmus, and W. Lee. "Definition of regions in human c-myc that are involved in transformation and nuclear localization." Mol Cell Biol 7 (5 1987): 1697-709.

Strasser, A., A. W. Harris, M. L. Bath, and S. Cory. "Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2." Nature 348 (6299 1990): 331-3.

Studzinski, G. P. and Z. S. Brelvi. "Changes in proto-oncogene expression associated with reversal of macrophage-like differentiation of HL 60 cells." J Natl Cancer Inst 79 (1 1987): 67-76.

Su, W., S. Jackson, R. Tjian, and H. Echols. "DNA looping between sites for transcriptional activation: self-association of DNA-bound Sp1." Genes Dev 5 (5 1991): 820-6.

Sundstrom, C. and K. Nilsson. "Establishment and characterization of a human histiocytic lymphoma cell line (U-937)." Int J Cancer 17 (5 1976): 565-77.

Suomalainen, H. A., L. C. Andersson, and J. Schroder. "Karyotypes in six leukaemia cell lines derived from patients with ALL." Hereditas 93 (1 1980): 85-91.

Swick, A. G., M. C. Blake, J. W. Kahn, and J. C. Azizkhan. "Functional analysis of GC element binding and transcription in the hamster dihydrofolate reductase gene promoter." Nucleic Acids Res 17 (22 1989): 9291-304.

Takeda, K., J. Minowada, and A. Bloch. "Kinetics of appearance of differentiation-associated characteristics in ML-1, a line of human myeloblastic leukemia cells, after treatment with 12-O-tetradecanoylphorbol-13-acetate, dimethyl sulfoxide or 1-beta-D-arabinofuranosylcytosine." Cancer Res 42 (12 1982): 5152-8.

Tapscott, S. J., R. L. Davis, M. J. Thayer, P. F. Cheng, H. Weintraub, and A. B. Lassar. "MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts." Science 242 (4877 1988): 405-11.

Tasanen, K., J. Oikarinen, K. I. Kivirikko, and T. Pihlajaniemi. "Promoter of the gene for the multifunctional protein disulfide isomerase polypeptide. Functional significance of the six CCAAT boxes and other promoter elements." J Biol Chem 267 (16 1992): 11513-9.

Tasset, D., L. Tora, C. Fromental, E. Scheer, and P. Chambon. "Distinct classes of transcriptional activating domains function by different mechanisms." Cell 62 (6 1990): 1177-87.

Tate, P. H. and A. P. Bird. "Effects of DNA methylation on DNA-binding proteins and gene expression." Curr Opin Genet Dev 3 (2 1993): 226-31.

Taylor, W. E. and E. T. Young. "cAMP-dependent phosphorylation and inactivation of yeast transcription factor ADR1 does not affect DNA binding." Proc Natl Acad Sci U S A 87 (11 1990): 4098-102.

Territo, M. C., T. Ganz, M. E. Selsted, and R. Lehrer. "Monocyte-chemotactic activity of defensins from human neutrophils." J Clin Invest 84 (6 1989): 2017-20.

Territo, M. C. and H. P. Koeffler. "Induction by phorbol esters of macrophage differentiation in human leukaemia cell lines does not require cell division." Br J Haematol 47 (3 1981): 479-83.

Thoma, F. and M. Zatchej. "Chromatin folding modulates nucleosome positioning in yeast minichromosomes." Cell 55 (6 1988): 945-53.

Thomas, A. E., J. Patterson, H. G. Prentice, M. K. Brenner, M. Ganczakowski, J. F. Hancock, J. K. Pattinson, H. A. Blacklock, and J. P. Hopewell. "Haemorrhagic cystitis in bone marrow transplantation patients: possible increased risk associated with prior busulphan therapy." Bone Marrow Transplant 1 (4 1987): 347-55.

Tice-Baldwin, K., G. R. Fink, and K. T. Arndt. "BAS1 has a Myb motif and activates HIS4 transcription only in combination with BAS2." Science 246 (4932 1989): 931-5.

Till, J. E. "Ontogeny of hematopoietic development and stem cells: introduction. pp. 3-4." In: Clarkson B. et al.. ed. Differentiation of normal and neoplastic hematopoietic cells. Cold Spring Harbor. Cold Spring Harbor Laboratory 5 (1978): 3-4.

Tobler, A., C. W. Miller, A. W. Norman, and H. P. Koeffler. "1,25-Dihydroxyvitamin D3 modulates the expression of a lymphokine (granulocyte-macrophage colony-stimulating factor) posttranscriptionally." J Clin Invest 81 (6 1988): 1819-23.

Tognoni, A., R. Cattaneo, E. Serfling, and W. Schaffner. "A novel expression selection approach allows precise mapping of the hepatitis B virus enhancer." Nucleic Acids Res 13 (20 1985): 7457-72.

Trautwein, C., P. van-der-Geer, M. Karin, T. Hunter, and M. Chojkier. "Protein kinase A and C site-specific phosphorylations of LAP (NF-IL6) modulate its binding affinity to DNA recognition elements." J Clin Invest 93 (6 1994): 2554-61.

Trentin, J. J. "Determination of bone marrow stem cell differentiation by stromal hemopoietic inductive microenvironments (HIM)." Am J Pathol 65 (3 1971): 621-8.

Trouche, D., M. Grigoriev, J. L. Lenormand, P. Robin, S. A. Leibovitch, P. Sassone-Corsi, and A. Harel-Bellan. "Repression of c-fos promoter by MyoD on muscle cell differentiation." Nature 363 (6424 1993): 79-82.

Tsai, S. Y., M. J. Tsai, and B. W. O'Malley. "Cooperative binding of steroid hormone receptors contributes to transcriptional synergism at target enhancer elements." Cell 57 (3 1989): 443-8.

Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada. "Establishment and characterization of a human acute monocytic leukemia cell line (THP-1)." Int J Cancer 26 (2 1980): 171-6.

Tsujimoto, Y., J. Cossman, E. Jaffe, and C. M. Croce. "Involvement of the bcl-2 gene in human follicular lymphoma." Science 228 (4706 1985): 1440-3.

Tsutsumi, K., K. Ito, and K. Ishikawa. "Developmental appearance of transcription factors that regulate liver-specific expression of the aldolase B gene." Mol Cell Biol 9 (11 1989): 4923-31.

Udvardy, A., E. Maine, and P. Schedl. "The 87A7 chromomere. Identification of novel chromatin structures flanking the heat shock locus that may define the boundaries of higher order domains." J Mol Biol 185 (2 1985): 341-58.

Umek, R. M., A. D. Friedman, and S. L. McKnight. "CCAAT-enhancer binding protein: a component of a differentiation switch." Science 251 (4991 1991): 288-92.

Urieli-Shoval, S., Y. Gruenbaum, J. Sedat, and A. Razin. "The absence of detectable methylated bases in Drosophila melanogaster DNA." Febs Lett 146 (1 1982): 148-52.

Urness, L. D. and C. S. Thummel. "Molecular interactions within the ecdysone regulatory hierarchy: DNA binding properties of the Drosophila ecdysone-inducible E74A protein." Cell 63 (1 1990): 47-61.

Valore, E. V. and T. Ganz. "Posttranslational processing of defensins in immature human myeloid cells." Blood 79 (6 1992): 1538-44.

Venturelli, D., M. T. Mariano, C. Szczylik, M. Valtieri, B. Lange, W. Crist, M. Link, and B. Calabretta. "Down-regulated c-myb expression inhibits DNA synthesis of T-leukemia cells in most patients." Cancer Res 50 (22 1990): 7371-5.

Villares, R. and C. V. Cabrera. "The achaete-scute gene complex of D. melanogaster: conserved domains in a subset of genes required for neurogenesis and their homology to myc." Cell 50 (3 1987): 415-24.

von-Melchner, H. and D. E. Housman. "The expression of neomycin phosphotransferase in human promyelocytic leukemia cells (HL60) delays their differentiation." Oncogene 2 (2 1988): 137-40.

Wang, H. M., M. Collins, K. Arai, and A. Miyajima. "EGF induces differentiation of an IL-3-dependent cell line expressing the EGF receptor." Embo J 8 (12 1989): 3677-84.

Wang, Y., R. Benezra, and D. A. Sassoon. "Id expression during mouse development: a role in morphogenesis." Dev Dyn 194 (3 1992): 222-30.

Wasylyk, B., C. Wasylyk, P. Flores, A. Begue, D. Leprince, and D. Stehelin. "The c-ets proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation." Nature 346 (6280 1990): 191-3.

Wasylyk, C., P. Flores, A. Gutman, and B. Wasylyk. "PEA3 is a nuclear target for transcription activation by non-nuclear oncogenes." Embo J 8 (11 1989): 3371-8.

Wasylyk, C. and B. Wasylyk. "Oncogenic conversion alters the transcriptional properties of ets." Cell Growth Differ 3 (9 1992): 617-25.

Watson, D. K., M. J. McWilliams, P. Lapis, J. A. Lautenberger, C. W. Schweinfest, and T. S. Papas. "Mammalian ets-1 and ets-2 genes encode highly conserved proteins." Proc Natl Acad Sci U S A 85 (21 1988): 7862-6.

Watson, D. K., F. E. Smyth, D. M. Thompson, J. Q. Cheng, J. R. Testa, T. S. Papas, and A. Seth. "The ERGB/Fli-1 gene: isolation and characterization of a new member of the family of human ETS transcription factors." Cell Growth Differ 3 (10 1992): 705-13.

Watson, R. J., C. Robinson, and E. W. Lam. "Transcription regulation by murine B-myb is distinct from that by c-myb." Nucleic Acids Res 21 (2 1993): 267-72.

Weber, B. L., E. H. Westin, and M. F. Clarke. "Differentiation of mouse erythroleukemia cells enhanced by alternatively spliced c-myb mRNA." Science 249 (4974 1990): 1291-3.

Wegner, M., Z. Cao, and M. G. Rosenfeld. "Calcium-regulated phosphorylation within the leucine zipper of C/EBP beta." Science 256 (5055 1992): 370-3.

Weinberger, C., C. C. Thompson, E. S. Ong, R. Lebo, D. J. Gruol, and R. M. Evans. "The c-erb-A gene encodes a thyroid hormone receptor." Nature 324 (6098 1986): 641-6.

Weintraub, H. "Histone-H1-dependent chromatin superstructures and the suppression of gene activity." Cell 38 (1 1984): 17-27.

Weintraub, H., V. J. Dwarki, I. Verma, R. Davis, S. Hollenberg, L. Snider, A. Lassar, and S. J. Tapscott. "Muscle-specific transcriptional activation by MyoD." Genes Dev 5 (8 1991): 1377-86.

Westin, E. H., R. C. Gallo, S. K. Arya, A. Eva, L. M. Souza, M. A. Baluda, S. A. Aaronson, and F. Wong-Staal. "Differential expression of the amv gene in human hematopoietic cells." Proc Natl Acad Sci U S A 79 (7 1982): 2194-8.

Weston, K. and J. M. Bishop. "Transcriptional activation by the v-myb oncogene and its cellular progenitor, c-myb." Cell 58 (1 1989): 85-93.

White, R. J. and S. P. Jackson. "The TATA-binding protein: a central role in transcription by RNA polymerases I, II and III." Trends Genet 8 (8 1992): 284-8.

Whyte, P. and R. N. Eisenman. "Dephosphorylation of the retinoblastoma protein during differentiation of HL60 cells." Biochem Cell Biol 70 (12 1992): 1380-4.

Wickstrom, E. L., T. A. Bacon, A. Gonzalez, G. H. Lyman, and E. Wickstrom. "Anti-c-myc DNA increases differentiation and decreases colony formation by HL-60 cells." In Vitro Cell Dev Biol 25 (3 Pt 1 1989): 297-302.

Wiedemann, L. M., G. E. Francis, R. F. Lamb, J. H. Burns, J. N. Winnie, E. D. MacKenzie, and G. D. Birnie. "Differentiation stage-specific expression of a gene during granulopoiesis." Leukemia 3 (3 1989): 227-34.

Wilde, C. G., J. E. Griffith, M. N. Marra, J. L. Snable, and R. W. Scott. "Purification and characterization of human neutrophil peptide 4, a novel member of the defensin family." J Biol Chem 264 (19 1989): 11200-3.

Wiley, S. R., R. J. Kraus, and J. E. Mertz. "Functional binding of the "TATA" box binding component of transcription factor TFIID to the -30 region of TATA-less promoters." Proc Natl Acad Sci U S A 89 (13 1992): 5814-8.

Williams, S. C., C. A. Cantwell, and P. F. Johnson. "A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro." Genes Dev 5 (9 1991): 1553-67.

Williams, T., A. Admon, B. Luscher, and R. Tjian. "Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements." Genes Dev 2 (12a 1988): 1557-69.

Winston, F. and M. Carlson. "Yeast SNF/SWI transcriptional activators and the SPT/SIN chromatin connection." Trends Genet 8 (11 1992): 387-91.

Wirth, T., L. Staudt, and D. Baltimore. "An octamer oligonucleotide upstream of a TATA motif is sufficient for lymphoid-specific promoter activity." Nature 329 (6135 1987): 174-8.

Wolf, D. and V. Rotter. "Major deletions in the gene encoding the p53 tumor antigen cause lack of p53 expression in HL-60 cells." Proc Natl Acad Sci U S A 82 (3 1985): 790-4.

Wolffe, A. P. and R. H. Morse. "The transcription complex of the Xenopus somatic 5 S RNA gene. A functional analysis of protein-DNA interactions outside of the internal control region." J Biol Chem 265 (8 1990): 4592-9.

Wolman, S. R., L. Lanfrancone, R. Dalla-Favera, S. Ripley, and A. S. Henderson. "Oncogene mobility in a human leukemia line HL-60." Cancer Genet Cytogenet 17 (2 1985): 133-41.

Wong, P. M., S. W. Chung, C. E. Dunbar, D. M. Bodine, S. Ruscetti, and A. W. Nienhuis. "Retrovirus-mediated transfer and expression of the interleukin-3 gene in mouse hematopoietic cells result in a myeloproliferative disorder." Mol Cell Biol 9 (2 1989): 798-808.

Workman, J. L. and R. G. Roeder. "Binding of transcription factor TFIID to the major late promoter during in vitro nucleosome assembly potentiates subsequent initiation by RNA polymerase II." Cell 51 (4 1987): 613-22.

Workman, J. L., I. C. Taylor, and R. E. Kingston. "Activation domains of stably bound GAL4 derivatives alleviate repression of promoters by nucleosomes." Cell 64 (3 1991a): 533-44.

Workman, J. L., I. C. Taylor, R. E. Kingston, and R. G. Roeder. "Control of class II gene transcription during in vitro nucleosome assembly." Methods Cell Biol 35 (1991b): 419-47.

Wu, C., P. M. Bingham, K. J. Livak, R. Holmgren, and S. C. Elgin. "The chromatin structure of specific genes: I. Evidence for higher order domains of defined DNA sequence." Cell 16 (4 1979a): 797-806.

Wu, C., Y. C. Wong, and S. C. Elgin. "The chromatin structure of specific genes: II. Disruption of chromatin structure during gene activity." Cell 16 (4 1979b): 807-14.

Xin, J. H., A. Cowie, P. Lachance, and J. A. Hassell. "Molecular cloning and characterization of PEA3, a new member of the Ets oncogene family that is differentially expressed in mouse embryonic cells." Genes Dev 6 (3 1992): 481-96.

Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach. "p21 is a universal inhibitor of cyclin kinases [see comments]." Nature 366 (6456 1993): 701-4.

Yamada, M. and K. Hashinaka. "[Molecular aspects of proliferation and differentiation of human myeloid leukemia cell HL-60]." Tanpakushitsu Kakusan Koso 36 (1 1991): 41-53.

Yamamoto, K. K., G. A. Gonzalez, P. Menzel, J. Rivier, and M. R. Montminy. "Characterization of a bipartite activator domain in transcription factor CREB." Cell 60 (4 1990): 611-7.

Yokoyama, K. and F. Imamoto. "Transcriptional control of the endogenous MYC protooncogene by antisense RNA." Proc Natl Acad Sci U S A 84 (21 1987): 7363-7.

Yoshinaga, S. K., C. L. Peterson, I. Herskowitz, and K. R. Yamamoto. "Roles of SWI1, SWI2, and SWI3 proteins for transcriptional enhancement by steroid receptors." Science 258 (5088 1992): 1598-604.

Zenke, M., A. Munoz, J. Sap, B. Vennstrom, and H. Beug. "v-erbA oncogene activation entails the loss of hormone-dependent regulator activity of c-erbA." Cell 61 (6 1990): 1035-49.

Zhang, L., V. Lemarchandel, P. H. Romeo, Y. Ben-David, P. Greer, and A. Bernstein. "The Fli-1 proto-oncogene, involved in erythroleukemia and Ewing's sarcoma, encodes a transcriptional activator with DNA-binding specificities distinct from other Ets family members." Oncogene 8 (6 1993): 1621-30.

Zhou, Q., P. M. Lieberman, T. G. Boyer, and A. J. Berk. "Holo-TFIID supports transcriptional stimulation by diverse activators and from a TATA-less promoter." Genes Dev 6 (10 1992): 1964-74.

Zobel, A., F. Kalkbrenner, G. Vorbrueggen, and K. Moelling. "Transactivation of the human c-myc gene by c-Myb." Biochem Biophys Res Commun 186 (2 1992): 715-22.

Zola, H., P. J. McNamara, H. A. Moore, I. J. Smart, D. A. Brooks, I. G. Beckman, and J. Bradley. "Maturation of human B lymphocytes--studies with a panel of monoclonal antibodies against membrane antigens." Clin Exp Immunol 52 (3 1983): 655-64.