# REGULATION OF MYELOID DIFFERENTIATION BY C-MYC AND ITS ANTAGONISTS

by

KEVIN M. RYAN

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

The Beatson Institute for Cancer Research,
CRC Beatson Laboratories,
Bearsden, Glasgow.

Faculty of Medicine,
University of Glasgow,
Glasgow.

July, 1996.

**Abstract.**

The enforced expression of c-*myc* is able to block the differentiation of myeloid cells. More recently, it has been shown that the correct functioning of c-Myc is not only dependent on the abundance of its dimerization partner, Max, but also on the levels of two other proteins which complex with Max, Mad and Mxi1. Analysis was made of the levels of their mRNAs, relative to those of c-*myc* mRNA, during the induced differentiation of myeloid leukaemic HL60 and U937 cells. This revealed that, the abundance of *mxi1* and *max* mRNA were largely maintained at levels comparable to those observed in untreated cells, but the levels of *max* mRNA were found to be markedly reduced at the very late stages of differentiation in HL60 cells induced by TPA. In contrast, the levels of *mad* mRNA were rapidly increased following differentiation induction by TPA. However, it was found that differentiation to granulocytes or monocytes/macrophages could also be achieved without a concomitant increase in the abundance of *mad* mRNA.

To further investigate the role of Mad during the differentiation of myeloid cells E-box DNA-binding was analysed. While Myc:Max complexes were lost rapidly following differentiation induction, no Mad-containing complexes were detected during differentiation to monocytes/macrophages, and those which were detected during granulocytic differentiation were only evident at the very late stages. The subsequent analysis of these Mad-containing complexes revealed that they were also unlikely to be able to antagonise c-Myc function as they did not contain Max.

In light of these findings, it was decided not to study these factors further, but to focus on the role played by c-*myc* in the control of differentiation *per se*. Although the mechanism by which c-Myc affects this process remains unknown, it is considered that it might result indirectly as an outcome of the continued cell-cycle progression invoked by c-Myc in cells which must growth arrest in order to differentiate. However, it is equally possible that a differentiation blockage occurs through a mechanism independent of c-Myc's involvement in cell-cycle progression. An analysis was therefore made of a differentiation-defective variant of the U937 cell line which, following treatment with TPA, does not differentiate, but rapidly ceases to proliferate, arresting at the $G_0/G_1$ phase of the cell cycle. Analysis during growth arrest revealed that, although this line down-regulated the expression of the Myc target gene, *ornithine decarboxylase*, it continued to express high levels of c-Myc protein, which retained the ability to bind its target sequence, CACGTG. Consequently, it was hypothesised that the continued expression of c-Myc in these cells may be responsible for their inability to differentiate in response to treatment with TPA. In agreement with this, down-regulation of the levels of c-Myc by antisense oligonucleotides directed against c-*myc* mRNA resulted in these cells acquiring characteristics of a terminally differentiated cell.

As Mad, Max and Mxi1 have all been shown to antagonize c-Myc function, an analysis was also made for mutations in the genes for these proteins. Both HL60 and HeLa cells were found to be hemizygous for *max*. Sequencing of the remaining allele in these cell lines revealed three nucleotide changes, when compared to the published sequence. However, these changes did not result in any amino acid change.

The relevance of all these findings to the regulation of myeloid differentiation, and in particular to the involvement of c-Myc in these processes, is discussed.

*For my parents, Sheila and Jim.*

*In acknowledgement of their immense support over the years, it is of them that I am truly proud.*

**Acknowledgements.**

I should first like to thank my supervisor, Dr. George Birnie, for all of his help during the course of this work. I am also deeply indebted to all the other members of the R3 group, both past and present, who have helped as both colleagues and friends during my stay at the Beatson. In addition, thanks must go to members of the R11 group, and in particular Dave, Billy and Sam, for only replying with a small sigh each time I appeared for even more advice or the 'occasional' reagent. Much appreciation is also extended to Bill Simpson, Noel Wigglesworth, Robert McFarlane, Liz Gordon and, in particular Auntie Anne and her front office friends, who have always responded with a smile even when presented with what to me seemed like a problem to large to overcome. I would also like to acknowledge the members of the Institute who, over the years, have agreed that the best place to discuss the merits of each others projects is over a beer(s) at The Brewery Tap. In this regard, I think, Andy Phillips (until he left for The States), Jeremy Fordham (until he married Ros), Lenny Wu (until he'd finished his first pint), Burkhard Haefner (until the food arrived), Rob Nibbs (until he met Amanda), Gerry Graham (until he realised it was North of the River) and Vivien Tannoch (no excuse) are all worthy of a particular mention. Outside the lab, I would also like to thank all the people at Gardner St. and the Freshneys for respectively, providing me with somewhere to stay and somewhere to write during my hour of need.

Last, but certainly not least, I would like to thank Vick for everything, and more, during the past two years and more than anything for standing by me during my grumpy phases of the past few months.

**Declaration.**

I declare that all the work in this thesis was performed personally unless otherwise acknowledged.

CONTENTS.

**DISCUSSION**

**CHAPTER 10: Discussion.**

**List of figures.**

**List of Tables.**

**Abbreviations.**

| | | |
|---|---|---|
| A | : | adenine. |
| A-MuLV | : | Abelson murine leukaemia virus. |
| Act D | : | actinomycin D. |
| AMV | : | avian myeloblastosis virus. |
| APS | : | ammonium persulphate. |
| ATP | : | adenosine triphosphate. |
| $A_x$ | : | absorbance/ x = wavelength. |
| bHLH | : | basic region, helix-loop-helix. |
| BMM | : | bone marrow macrophages. |
| bp | : | base pair (s). |
| C | : | cytosine. |
| c- | : | cellular. |
| C/EBP | : | CCAAT/enhancer binding protein. |
| cDNA | : | complementary deoxyribonucleic acid. |
| cm | : | centimetre. |
| cpm | : | counts per minute. |
| Da | : | dalton (s). |
| DEPC | : | diethylpyrocarbonate. |
| DFMO | : | α-difluoromethylornithine. |
| $dH_2O$ | : | de-ionised water. |
| DHSS | : | DNAse I hypersensitive site. |
| DMSO | : | dimethyl sulphoxide. |
| DNA | : | deoxyribonucleic acid. |
| DNAse | : | deoxyribonuclease. |
| dNTP | : | 3' deoxyribonucleoside 5' triphosphate. |
| DTT | : | dithiothreitol. |
| eBL | : | endemic Burkitt's lymphoma. |
| EBV | : | Epstein-Barr virus. |
| ECL | : | enhanced chemiluminescence. |
| EDTA | : | ethylenediaminetetra-acetic acid, disodium salt. |
| EMSA | : | elctrophoretic mobility shift assay. |
| g | : | gram (s). |
| G | : | guanine. |
| GAPDH | : | glyceraldehyde phosphate dehydrogenase. |
| HEPES | : | N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid). |
| HFF | : | human foreskin fibroblasts. |
| HLH | : | helix-loop-helix. |
| HPV | : | human papilloma virus. |
| hr | : | hour (s). |
| IL-3 | : | interleukin 3. |
| Inr | : | initiator element. |
| KAc | : | potassium acetate. |
| kb | : | kilobase (s). |
| LMP 1 | : | Epstein-Barr virus latent membrane protein 1. |
| LZ | : | leucine zipper. |
| μ | : | micro. |

| | | |
|---|---|---|
| m | : | milli. |
| M | : | molar. |
| M-CSF | : | macrophage colony stimulating factor. |
| mA | : | milliamps. |
| MEL | : | murine erythroleukaemia. |
| min | : | minute (s). |
| mm | : | millimetres. |
| Mo-MuLV | : | Moloney murine leukaemia virus. |
| MOPS | : | 3-(N-morpholino) propanesulphonic acid. |
| mRNA | : | messenger ribonuleic acid. |
| Myc-ER | : | c-Myc-oestrogen receptor fusion protein. |
| n | : | nano. |
| NaAc | : | sodium acetate. |
| NBT | : | nitroblue tetrazolium. |
| NSE | : | non-specific esterase. |
| OAG | : | 1-oleoyl-2-acetyl-*sn*-glycerol. |
| °C | : | degrees Celsius. |
| ODC | : | ornithine decarboxylase. |
| oligo | : | oligonucleotide. |
| PAGE | : | polyacrylamide gel electrophoresis. |
| PBS | : | phosphate-buffered saline. |
| PCR | : | polymerase chain reaction. |
| PKC | : | protein kinase C. |
| PMSF | : | phenylmethylsulphonyl fluoride. |
| PNK | : | polynucleotide kinase. |
| Rb-1 | : | retinoblastoma protein. |
| RNA | : | ribonucleic acid. |
| RNAse | : | ribonuclease. |
| rpm | : | revolutions per minute. |
| RT | : | reverse transcription. |
| SDS | : | sodium dodecyl sulphate. |
| sec | : | second (s). |
| SSC | : | sodium chloride, sodium citrate. |
| SSPE | : | sodium chloride, sodium phosphate, ethylenediaminetetra-acetic acid. |
| SV40 | : | simian virus 40. |
| T | : | thymine. |
| TEMED | : | tetramethylenediamine. |
| TPA | : | 12-O-tetradecanoyl-phorbol-13-acetate. |
| Tris | : | 2-amino-2-(hydroxymethyl) propane-1,3-diol. |
| U | : | units. |
| UV | : | ultra violet. |
| V | : | volts. |
| v- | : | viral. |
| v/v | : | volume for volume. |
| Vit D3 | : | 1-α,25-dihydroxyvitamin D3. |
| w/v | : | weight for volume. |
| wt- | : | wild-type. |

# INTRODUCTION

# INTRODUCTION

## CHAPTER 1: Biochemistry of the mammalian *myc* genes.

### 1.1 Background and historical perspectives.

The majority of the work on the *myc* gene family has focused on three members which, when activated, have been shown to be important in the generation of various human malignancies (Field and Spandidos 1990; Zimmerman and Alt 1990; Spencer and Groudine 1991). The most studied of these, c-*myc*, was first discovered through its homology to the transforming gene (v-*myc*) of the avian myelocytomatosis virus MC29 (Vennstrom et al. 1982). The other two, N-*myc* and L-*myc*, were later discovered through their homology to v-*myc* in the amplified sequences of neuroblastoma cells (Schwab et al. 1983) and a small cell lung tumour (Nau et al. 1985) respectively. In addition to these three proto-oncogenic family members, two other *myc* genes S-*myc* and B-*myc* have also been identified. Despite being only partially characterised, these two genes appear highly interesting since they are different to c-*myc*, L-*myc* and N-*myc* in that they suppress malignant transformation (Table 1).

In a variety of species the *myc* proto-oncogenes have been shown to be activated by gene amplification (Dalla-Favera et al. 1982), chromosomal translocation (Magrath 1990), proviral insertion (Payne et al. 1982), and retroviral transduction (Neil et al. 1987) (Table 2). However, it is most frequently reported that the level of the c-Myc protein is elevated in many tumour types by a mechanism that is none of the above and is as yet not understood (Erisman et al. 1985). Early research into the proteins implicated their expression to be associated with cell-cycle progression (Kelly and Siebenlist 1986) and incompatible with terminal differentiation in a variety of cell lineages (Ingvarsson 1990; Luscher and Eisenman 1990; Evan and Littlewood 1993). In addition, c-*myc* was shown to cause cellular

immortalization (Penn et al. 1990) and was able to co-operate with an activated *ras* gene in the transformation of rat embryo fibroblasts cells (Land et al. 1983a).

| ***myc* gene** | **Functional Characteristics** | **Ref.** |
|---|---|---|
| **c-*myc*** | Cellular transformation<br>Apoptosis<br>Continued cell-cycle progression | Luscher and Eisenman (1990)<br>Ingvarsson (1990)<br>Penn et al. (1990)<br>Evan et al. (1992) |
| **L-*myc***<br>**N-*myc*** | Cellular transformation<br>Continued cell-cycle progression | Luscher and Eisenman (1990)<br>Ingvarsson (1990)<br>Penn et al. (1990) |
| **S-*myc*** | Growth suppression<br>Apoptosis | Sugiyama et al. (1989)<br>Asai et al. (1994) |
| **B-*myc*** | Inhibition of neoplastic transformation | Resar et al. (1993) |
| **P-*myc***<br>**L-*myc* ψ** | pseudogenes | De Pinho et al. (1991) |

**Table 1. Summary of the mammalian *myc* genes described to date**

In 1985, Eisenman et al. (1985) established that, in cells containing active c-Myc or v-Myc protein, the majority of the protein was associated with a nuclear fraction, termed the "matrix lamin". This observation was later substantiated by the identification of a domain within the c-Myc protein that was found to be effective as

a nuclear localization signal (Dang and Lee 1988). As a result of these findings, two schools of thought were generated as to how the Myc proteins brought about phenotypic change. It was proposed that they either had a direct role in the DNA replication machinery (Studzinski et al. 1986) or were involved in the transcriptional control of genes involved in cellular replication (Kaddurah-Daouk et al. 1987).

| **Mechanism of *c-myc* activation** | **Effects** | **Incidence** |
|---|---|---|
| **Amplification of gene** | Increased Myc protein abundance. | Gastric adenocarcinoma, small cell lung carcinoma, glioblastoma, carcinoma of breast, carcinoma of colon, plasma cell leukaemia, promyelocytic leukaemia, granulocytic leukaemia. |
| **Proviral insertion** | Deregulated expression of *myc* by viral long terminal repeat | Leukaemia resulting from infection by: avian leucosis virus, Moloney murine leukaemia virus |
| **Retro-viral transduction** | Deregulated expression of viral Gag-Myc fusion protein. | Feline leukaemia viruses e.g. GT3 & FTT<br>Avian leukaemia viruses e.g. MC29 & MH2 |
| **Chromosomal translocations** | Deregulated expression of full-length or truncated *myc*. | Burkitt's lymphoma<br>Mouse plasmacytoma |

**Table 2: Incidence, mechanism, and effects of *myc* activation. (Abstracted from Marcu et al. (1992)).**

Evidence for a role in the control of gene expression grew when the sequence of the c-Myc protein was shown to contain a series of motifs which were similar to ones previously described for known transcription factors. Leucine zipper (LZ) motifs, like those found in the onco-proteins v-Fos and v-Jun, were the first to be identified and were shown to be located in the extreme C-terminus of the protein (Landschulz et al. 1988) (Figure 1). Subsequently, immediately upstream of the leucine zipper motif, similarity was then found to a second domain termed the "helix-loop-helix" (HLH) motif (Luscher and Eisenman 1990). This domain had already been identified in a number of transcription factors including the immunoglobulin enhancer binding proteins E12 and E47 (Murre et al. 1989). However, although both of these motifs had previously been shown to be involved in the formation of transcription factor complexes, attempts to detect complexes in which c-Myc was either homo or hetero-oligomerized proved fruitless. In spite of this, further studies then revealed that the Myc proteins also contained a tract of basic amino acids upstream of the helix-loop-helix motif (Figure 1). This "basic region" motif had been previously identified in the myogenic transcription factor MyoD and was found to be the region involved in determining sequence-specific DNA binding (Davis et al. 1990). As a final piece of indirect evidence for Myc family members being involved in the control of gene expression, it was shown that a region at the N-terminus of c-Myc (Figure 1) had the ability to act as a transcriptional transactivator. Fusion genes were constructed in which the c-*myc* N-terminal region was fused to the DNA binding domain of the gene for the yeast transcription factor GAL 4. This construct was then transiently co-transfected into cells, with a reporter construct containing the GAL 4 DNA binding site upstream of the chloramphenicol acetyl transferase (CAT) gene. The results of these experiments indicated that the transactivation potential of the fusion protein was both potent and highly specific for the N-terminal region of c-Myc (Kato et al. 1990).

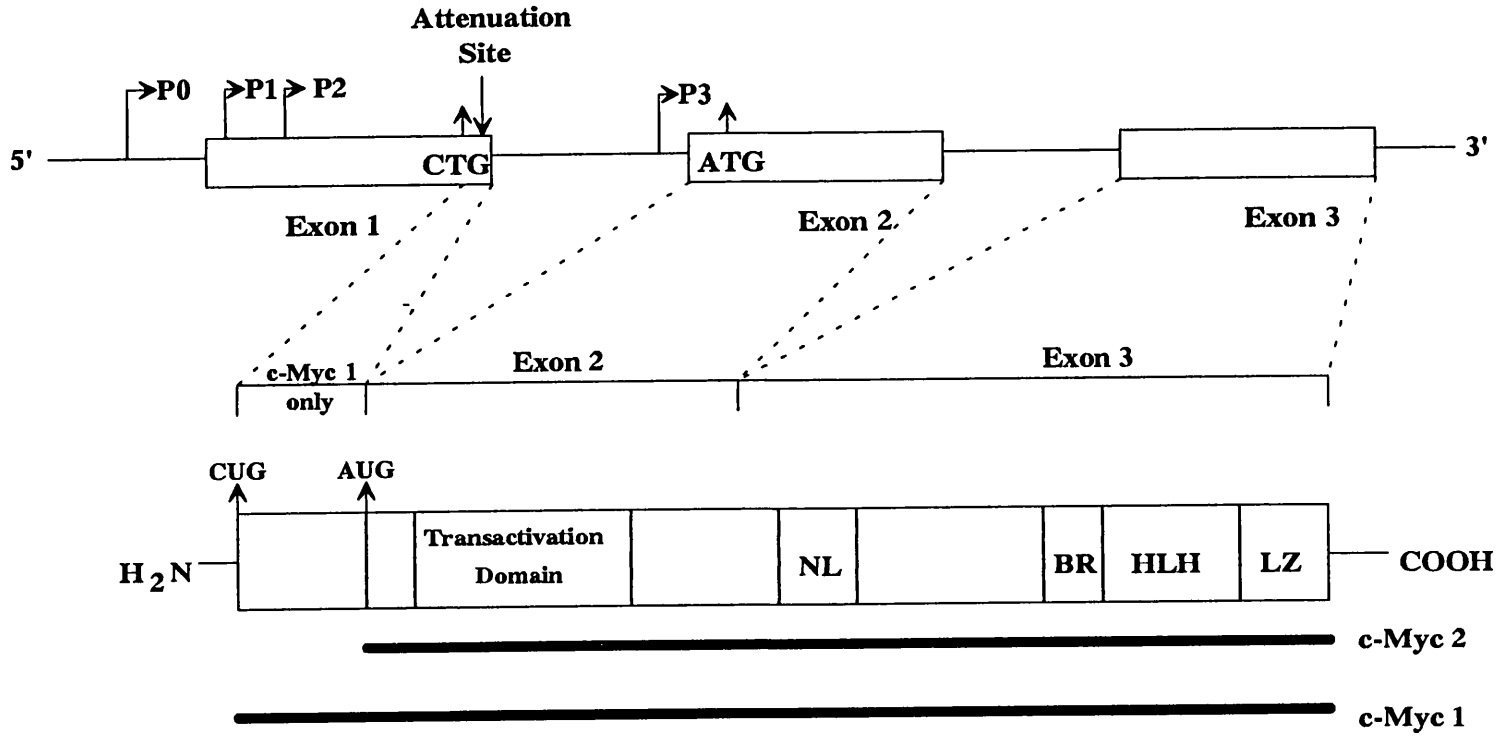



Figure 1:*Schematic representation of the structure of the human c-myc gene and the two resultant protein products c-Myc 1 and c-Myc 2.*

Sites of the genes promoters are indicated as P0, P1, P2 and P3. The translation initiation sites of the two proteins are shown as the codons of the initial amino acid. i.e. CTG(CUG) and ATG(AUG). NL, nuclear localization; BR, basic region, HLH, helix-loop-helix; LZ, leucine zipper.

## 1.2 A role as a transcription factor.

### 1.2.1 *Myc as an activator.*

As outlined above (section 1.1), many lines of indirect evidence accumulated to indicate that the Myc proteins functioned as transcription factors. However, problems arose in confirming this theory when it was found that Myc proteins could only form complexes with DNA at very high protein concentrations, implying that such interactions may not be physiologically significant (Dang et al. 1989). It was therefore considered that Myc might require interaction with a second protein in order to achieve this. With this in mind, Blackwood and Eisenman screened a baboon expression library with a c-Myc protein labelled with $^{125}I$. Subsequent sequencing of hybridising colonies identified a small, novel protein, which they named Max (Blackwood and Eisenman 1991) (Figure 2). Analysis of this protein revealed that it was similar to Myc in that it also contained bHLH and LZ motifs and was therefore considered to be a possible dimerization partner. Indeed, when assayed *in vitro* it was found that Max was able to form dimeric complexes with c-Myc, L-Myc and N-Myc, and at much lower protein concentrations than had previously been required to achieve Myc homodimerization (Blackwood and Eisenman 1991). In addition, when a complex of c-Myc and Max was used to select preferred DNA sequences from a pool of partially randomised oligonucleotides, it was found that the dimer had specific DNA binding activity for the sequence CACGTG (Solomon et al. 1993). Further studies of this sequence using electrophoretic mobility shift assays (EMSA) showed that, although homodimers of Max were able to bind without the presence of Myc, appreciable binding of Myc was dependent on it forming a heterodimeric complex with Max (Prendergast et al. 1991).

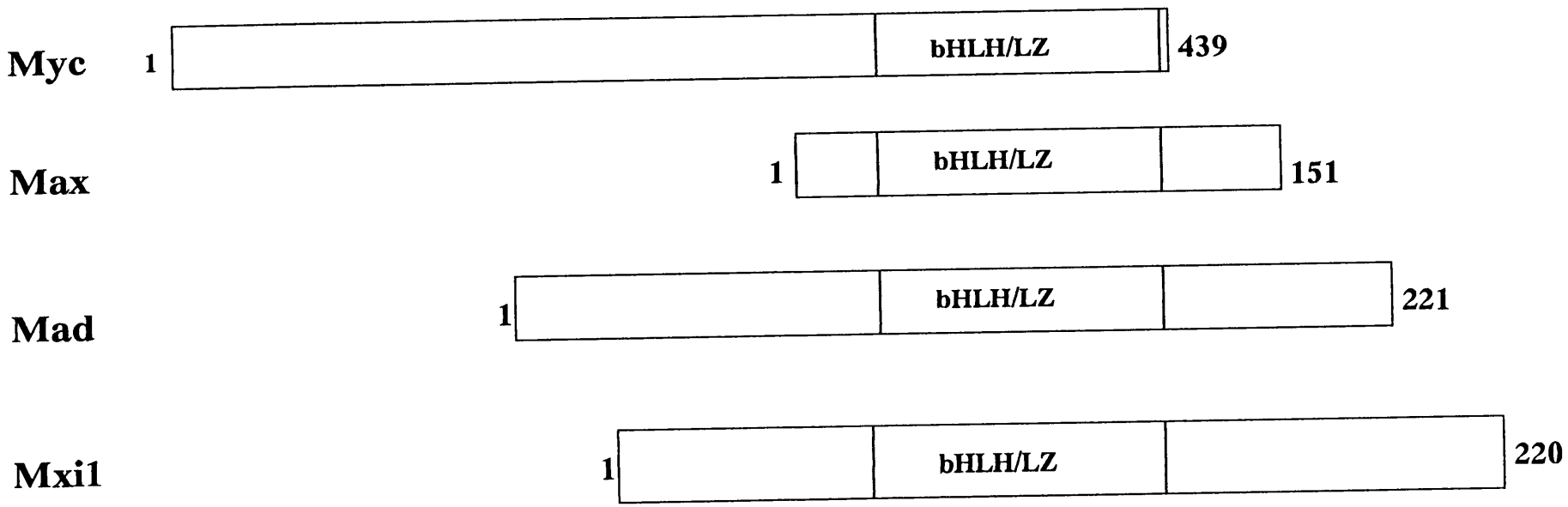



Figure 2: *Alignment of Myc, Max, Mad and Mxi1.*

Domains which are common to each protein and involved in heterodimerization are shown as bHLH/LZ. bHLH, basic helix-loop-helix domain; LZ, leucine zipper.

Since the CACGTG motif had previously been identified as the binding site for other transcription factors containing basic region and helix-loop-helix (bHLH) motifs, it seemed encouraging that this might be the transcription target for Myc. This was analysed by a yeast reporter gene assay, in which Myc and Max were co-expressed with a β-galactosidase gene under the control of a basal promoter linked to the CACGTG sequence. When c-Myc and Max were co-expressed in this system, there was a considerable increase in the activity of the β-galactosidase gene (Amati et al. 1992). The level of activity was dependent not only on the level of Myc, but also on the presence of the domains which had previously implicated Myc as a transcription factor. Although it was not a surprise that expression of c-Myc alone did not activate the reporter construct, it was intriguing to find that even though Max:Max homodimers bound to the CACGTG sequence they too did not activate the β-galactosidase gene (Amati et al. 1992)(Figure 3). Titration experiments revealed that if the Max protein was expressed at high levels in conjunction with c-Myc, the β-galactosidase activity observed when c-Myc and Max were expressed in roughly equivalent amounts was repressed. Although it was shown that Max preferentially formed a heterodimer with Myc as opposed to a homodimer (Blackwood and Eisenman 1991; Prendergast et al. 1991)(Figure 3), this created a situation whereby the activity of genes regulated by Myc would not only be dependent on the levels of Myc, but also upon the levels of Max. The most likely explanation for the apparent lack of transcriptional activity by Max:Max homodimers has been given by Kato et al. (1992). In experiments designed to identify a transactivation domain in the Max protein, they constructed fusion proteins linking regions of Max with the DNA binding domain of GAL 4. When assayed for their ability to transactivate a reporter gene linked to the GAL 4 DNA binding site it was found that, unlike the Myc transactivation domain, no region of Max was effective in activating the gene's expression.

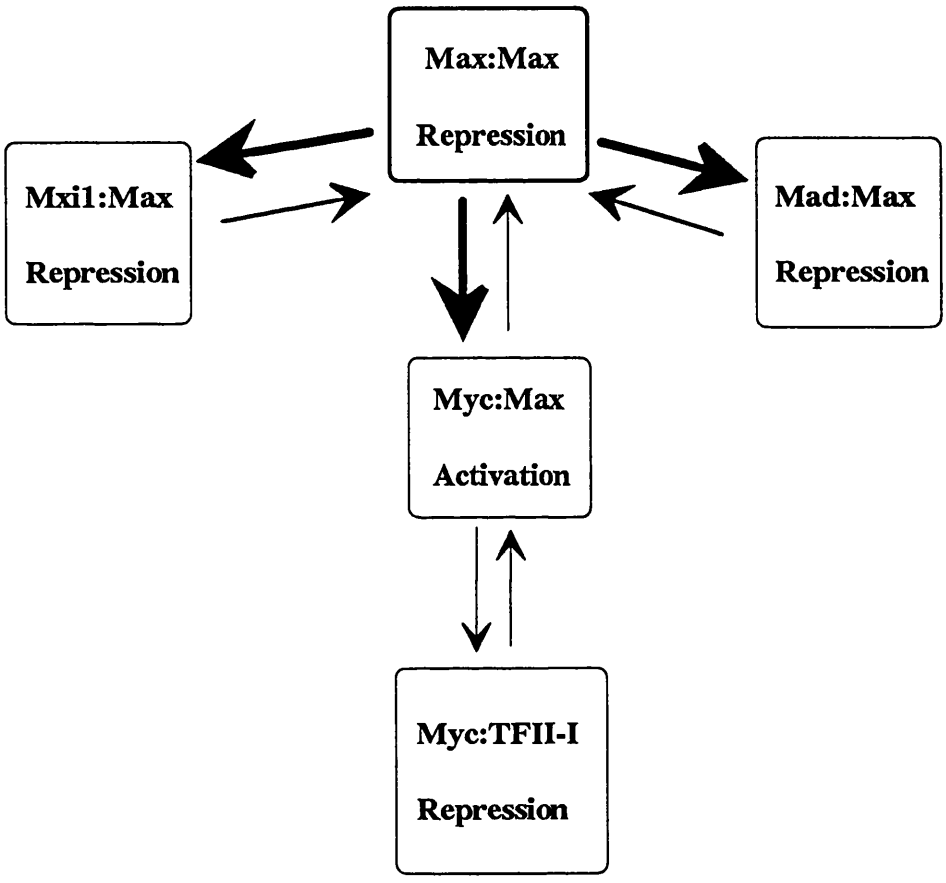



Figure 3: *Transcriptional consequences of Myc-associated complexes.*

Arrow strength indicates preferential complex formation.

Although the CACGTG motif is present in the promoter regions of various genes, the search for Myc activated genes has been somewhat without reward. In an attempt to address this problem Eilers et al. (1991) generated a fusion protein between c-Myc and a portion of the oestrogen receptor. When introduced into mammalian cells, the activity of the exogenous Myc (Myc-ER) protein becomes dependent on the presence of the steroid hormone β-oestradiol. cDNA libraries were then generated corresponding to mRNA species from control cells and from cells treated with β-oestradiol for 24 hr. Comparison of these two libraries identified a message which was induced by Myc as encoding α-prothymosin. Subsequent studies revealed that the gene could be activated by Myc in the absence of protein synthesis and that its activation was dependent upon the integrity of a CACGTG sequence found within the first intron of the gene (Gaubatz et al. 1994). As might be expected for a gene regulated by c-Myc, expression of α-prothymosin has been associated with proliferating cells (Eschenfeldt et al. 1986; Gomez-Marquez et al. 1989) and it is expressed in nearly all tissues (Clinton et al. 1989). However, a role for α-prothymosin is yet to be found and as a result its involvement in the phenotypes brought about by activated expression of Myc proteins remains a mystery.

A more appealing target for regulation by c-Myc is the enzyme ornithine decarboxylase (ODC). This enzyme catalyses the rate-limiting step in the production of polyamines (Tabor and Tabor 1984). Expression of *ODC* is regulated in a cell-cycle-specific manner and is greatly elevated upon cellular transformation (Pegg 1986). In addition, constitutive over-expression of *ODC* is able to bring about morphological transformation and its enhanced activity has been shown to be essential for transformation induced by the v-*src* oncogene (Auvinen et al. 1992). The observation that the *ODC* gene contains two CACGTG sequences led to the speculation that it too may be regulated by Myc. Studies using the Myc-ER chimeric protein revealed that *ODC* was indeed activated by Myc (Wagner et al.

1993) and that its activation was dependent on the two Myc:Max binding sites within the first intron (Bello-Fernandez et al. 1993). Further to these studies, it must be pointed out that while CACGTG is considered the primary target for the Myc:Max heterodimeric complex, Blackwell et al. (1993) have also demonstrated binding to a second canonical site (CATGTG) as well as to a series of non-canonical sequences. However, unlike the CACGTG sequence, the significance of these additional sites to the regulation of any genes by Myc:Max is yet to be established.

### 1.2.2 *Myc as a repressor.*

The search for direct targets of c-Myc has perhaps been hindered by the relative difficulty of identifying genes which may be inactivated as opposed to activated by the protein. In this regard it is well established that the number of genes which have been shown to be repressed by Myc expression in *in vitro* reporter gene studies (Kaddurah-Daouk et al. 1987) or which are repressed in Myc-transformed cells, is in excess of those which have been reported to be activated. Recently, upon the discovery that c-Myc could interact with TFII-I (Roy et al. 1993) (Figure 4), a transcription initiation factor that activates core promoters through a sequence termed the initiator element (Inr) (Smale and Baltimore 1989), studies were undertaken to determine if Myc might participate in transcriptional control involving this protein. *In vitro* transcription assays using the adenovirus major late promoter revealed that, when introduced into a system involving activation by TFII-I, Myc protein was effective in bringing about transcriptional repression (Figure 3). Moreover, these effects were shown to be specific for TFII-I and were not apparent when c-Myc was introduced into systems being initiated by other factors e.g. TFII-A (Roy et al. 1993). Subsequent analysis of the *cyclin* D1 gene, which had previously been shown to be repressed in Myc-transformed cells, indicated that the repression of this gene by Myc might also be mediated through

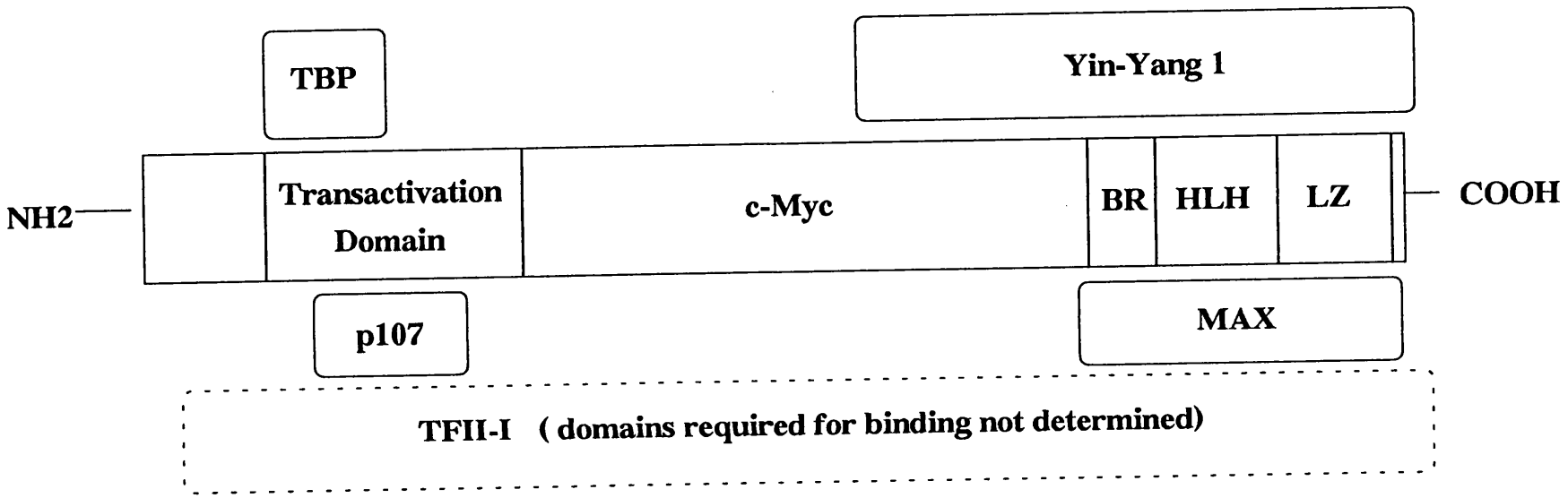



Figure 4: *Representation of the sites of interaction of proteins known to bind c-Myc.*

BR, basic region; HLH, helix-loop-helix; LZ, leucine zipper; TBP, TATA binding protein component of the transcription initiator, TFII-D.

TFII-I (Phillip et al. 1994). One might consider that the apparent repression of *cyclin* D1 by c-Myc seems paradoxical bearing in mind the involvement that the Cyclin is known to play in cell-cycle progression. In this regard, it must be pointed out that an independent study has implicated c-Myc in the activation of *cyclin* D1 transcription (Daksis et al. 1994). Further work is therefore required to establish the exact role, if any, that Myc is playing in the regulation of this gene.

Although a model for Myc action involving transcription repression is at first appealing, the probability of it being involved in the establishment of a transformed phenotype appears unlikely. Mutational studies of c-Myc and Max by Amati et al. (1993a) have indicated that cell-cycle progression and transformation by Myc proteins requires dimerization with Max. In contrast, inhibition of transcription through interaction with TFII-I occurs independently of Max and is refractory to forced expression of the Max protein (Phillip et al. 1994). Also, when mutants of the c-Myc protein were analysed for their ability to repress the promoter of the *cyclin* D1 gene, a deletion mutant involving amino acids 92-106 was ineffective in bringing about transcriptional repression (Phillip et al. 1994), even though this region was previously shown to be dispensable during Myc-induced transformation of RAT1A cells (Stone et al. 1987). However, these observations do not mean that transcriptional repression through TFII-I is artifactual. It would be naive to assume that the only transcriptional targets of c-Myc are those involved in transformation. For example, the ability of Myc proteins to inhibit processes of differentiation have not as yet been shown to be dependent on Max, and are therefore a possible Myc function in which TFII-I may be involved.

### 1.3 Other proteins which bind to Myc.

An emerging theme arising from the study of transformed cells is that onco-proteins are frequently found to be associated with proteins having tumour

suppressor or anti-proliferative function. Classically this observation comes from the analysis of the cellular proteins which co-precipitate with the transforming proteins of DNA tumour viruses. For example, the Adenovirus E1A protein and the human papillomavirus type 16 and 18 E7 protein have been shown to associate with the Retinoblastoma protein (Rb-1) (Dyson et al. 1989a; Whyte et al. 1988), while the respective E1B and E6 proteins of these viruses are believed to form complexes with p53 (Werness et al. 1990; Sarnow et al. 1982). In addition, it is now becoming a frequent observation that, within the normal cellular environment, proteins having potential oncogenic activity are often found complexed to those with known tumour suppressor function, for example, the association between the cyclin dependent kinase 4 protein and the recently discovered tumour suppressor protein mts1/p16 (Serrano et al. 1993). It has now become apparent that the c-Myc protein forms such an interaction with the Retinoblastoma family member p107 (Beijersbergen et al. 1994; Gu et al. 1994) (Figure 4). This protein had previously been shown to form complexes *in vivo* with the adenovirus E1A protein (Whyte et al. 1989), the SV40 virus large T antigen (Dyson et al. 1989b) and the human papillomavirus type 16 E7 protein (Davies et al. 1993). Although it was known that this interaction formed an important part of the transforming ability of these viruses, its consequences for cellular homeostasis were not understood. Since the interaction between p107 and the N-terminus of c-Myc results in the inactivation of the transactivation potential of the Myc protein, it seems feasible that binding of p107 by DNA tumour viruses might facilitate transformation by freeing the N-terminus of c-Myc and allowing the protein to interact with the TATA binding protein (Maheswaren et al. 1994) (Figure 4). This would then allow Myc to transactivate its target genes. With respect to how this might be important to the establishment of tumours *in vivo*, the study included an analysis of mutated c-Myc proteins derived from Burkitt's lymphoma cell lines. Although these proteins retained an ability to

form complexes with p107, they were unlike wild-type Myc in that their transactivation potential was unaffected by the interaction.

Perhaps more puzzling than the interaction with p107 is the report that c-Myc also interacts with the zinc finger protein Yin-Yang-1 (YY-1) (Shrivastava et al. 1993) (Figure 4). Depending on context, YY-1 has been shown to activate (Riggs et al. 1991), repress (Shi et al. 1991) and initiate transcription (Seto et al. 1991). However, when the YY-1 protein is bound to c-Myc both its ability to activate and repress transcription are reported to be inhibited (Shrivastava et al. 1993). Bearing in mind the work summarised above regarding the association between Myc and p107, it is interesting to note that among the targets for repression by YY-1 are the promoters of the adeno-associated virus P5 (Seto et al. 1991) and the human papillomavirus type (HPV) type 18 (Bauknecht et al. 1992). This therefore might be a possible reason for a negative regulator of Myc, i.e. p107, being a target for inhibition by HPV E7 (Davies et al. 1993). Although this theory is highly speculative it is somewhat reminiscent of the effect of the Adenovirus E1A protein upon the interaction between the Rb-1 protein and the transcription factor E2F (Shirodkar et al. 1992). Binding of Rb-1 by E1A results in the subsequent release of E2F such that it not only activates transcription of cellular genes involved in replication, but it also causes activation of the Adenovirus E2 promoter (Kovesdi 1986). Since little is as yet known about the function and mechanism of action of YY-1 more work is required to establish the significance of its association with c-Myc and the effect this may have on the replication of DNA tumour viruses.

### 1.4 Other proteins which bind to Max.

It has been shown more recently that Max also form complexes with two other proteins, Mad (Ayer et al. 1993) and Mxi1 (Zervos et al. 1993)(Figure 2). Whilst Mad:Max and Mxi1:Max complexes are similar to Myc:Max in that they

have also been shown to bind the CACGTG motif, they are more like the Max:Max homodimer in that they are thought to act as transcription repressors (Figure 3). However, it has more recently been shown that in contrast to the Max:Max homodimer, Mad:Max and Mxi1:Max are only effective in transcription repression as a ternary complex with homologues of the yeast transcription repressor Sin3 (Ayer et al. 1995; Schreiber-Agus et al. 1995). Although the role that these two new complexes may play within the cell is yet to be established, it is reasonable to assume that, since they are in competition with Myc for available Max protein, that the levels or activities of Mad or Mxi1 may have a bearing on the ability of Myc to transactivate the promoters of its target genes . In fact, when these two genes were over-expressed in cells transformed by c-*myc* and *ras*, the number of cells scoring positive in an assay of transformation was greatly reduced (Lahoz et al. 1994). In this regard, it could be quite possible that loss or mutation of either *mad* or *mxi1* would result in release of Max protein, which would then be available to co-operate with Myc in situations where the abundance of Myc was elevated. However, since these genes have only recently been identified there has only been one report to date that would indicate that these genes might act as tumour suppressors in the genesis of human cancer. Eagle et al. (1995) investigated the possibility that the *mxi1* gene, which maps to 10q24-q25, might be involved in a characteristic deletion of this area that occurs in a small number of cases of carcinoma of the prostate. The study reported that in five tumours analysed, the *mxi1* locus was indeed reduced to hemizygosity. In addition, in four of the five cases the remaining allele involved was also mutated. This initial finding is highly provocative and it can now be considered an appropriate time to investigate whether *mxi1*, and *mad*, are targets for mutation in other tissues.

## 1.5 Regulation of the abundance of the c-Myc protein.

### 1.5.1 *Transcriptional control.*

It is reasonable to assume that the primary control of the abundance of a protein is largely determined by transcriptional initiation. Workers on Myc have not ignored this fact and as a result a large amount of information has accumulated on the regulation of the c-*myc* promoter. A complex story has evolved in which the gene is controlled by not just one promoter, but in fact by four. The two major promoters, P1 and P2 (Figure 1) contribute 75-90% and 10-25% of the cytoplasmic c-*myc* mRNAs, respectively (Stewart et al. 1984; Taub et al. 1984). Approximately 1500bp downstream of P1 and P2 and close to the translation start site lies the third promoter P3. This promoter is thought to be less significant than P1 and P2, as it only contributes about 5% of c-*myc* mRNAs (Ray and Robert-Lesenges 1989). Of similar activity, but only present in the human c-*myc* gene, is a fourth promoter (P0) (Bentley and Groudine 1986), which is located around 600bp upstream of the major sites, P1 and P2 (Figure 1). The significance of having the four promoters is as yet unknown, but their differential usage has been observed in many cell lineages and upon the gene's deregulated expression (Siebenlist et al. 1984).

Analysis of this 5' region of the c-*myc* gene for DNase I hypersensitive sites (DHSS) revealed multiple regions of potential protein interaction (reviewed by Marcu et al. 1992). One region of particular note is a binding site which lies -65 to -58bp upstream of the P2 promoter. Deletion of this region has indicated that it is essential for the basal activity of P2 (Lipp et al. 1989) and also for the activation of c-*myc* induced by the adenovirus E1A protein (Hiebert et al. 1989). Studies of this region led to the discovery that this region was bound by the cell-cycle-regulated transcription factor E2F (Thalmeier et al. 1989). In light of this finding, it is encouraging that it has since been shown that activation of E2F

is sufficient to direct cell-cycle progression (Johnson et al. 1993), a characteristic that has also been attributed to the c-Myc protein. However, it is yet be established whether this function of the E2F protein is, at least in part, mediated through activation of c-*myc* transcription.

Whilst it is perhaps not very surprising that a region of the c-*myc* promoter is controlled by a cell-cycle-regulated transcription factor, studies of other regions have proved to be more intriguing. The analysis of a further DHSS situated approximately 130bp upstream of P2 revealed a region that was highly important for P1 activation as well as being modestly involved in the activation of P2 (Hay et al. 1987). Partial fractionation of HeLa cell nuclear proteins by Postel et al. (1989) revealed a DNA binding activity that was specific for this region and which they termed PuF. The surprising twist came when the binding site for PuF was used to screen a HeLa cell cDNA expression library. The subsequent sequence analysis of a hybridising clone revealed that it was identical to nm23-H2 (Postel et al. 1993). Surprisingly, the protein product of this gene is one polypeptide of the NM23 nucleotide diphosphate kinase, an enzyme which has been implicated in suppressing the metastatic spread of certain tumours (Delarosa et al. 1995). Admittedly, it is difficult to comprehend why a suppressor of metastatic disease should be involved in the activation of a gene that is frequently elevated in the late stages of human cancer. Further work is therefore required to establish the role that this protein is playing not only in the regulation of c-*myc*, but also in the establishment of a metastatic state.

### 1.5.2 *Other levels of control.*

Perhaps disheartening to the investigators involved in studying the transcriptional control of c-*myc* was the finding by Bentley and Groudine (1986) that the initial control of c-*myc* mRNA levels during induced differentiation of

myeloid leukaemic cells was by transcriptional attenuation. Using nuclear run-on analysis they found that the ratio of exon 1 transcription relative to that of exon 2 was increased from 3 to 15 following induction of the differentiation programme. Subsequent analysis of the sequences involved in this process identified a 95bp segment, located at the 3' end of exon 1 (Figure 1), to be the site of the premature termination (Bentley and Groudine 1988). However, when this site was analysed for its ability to attenuate the transcription which was directed by other promoters its effectiveness was highly variable (Bentley and Groudine 1988). In this regard, it has since been shown the sequences responsible for the premature termination are not located at the site of attenuation, but are in fact found proximal to the P2 promoter (Krumm et al. 1992). In this study, it was postulated that at this site the RNA polymerase might undergo a pause at which point it is modified in a way which determines how it will respond at the site of attenuation.

Although a large number of data have accumulated as to the transcriptional regulation of the c-*myc* gene, it must be pointed out that the mRNA is also subject to post-transcriptional control. Both the mRNA and protein have been shown to have extremely short half-lives of 15 min (Dani et al. 1984) and 30 min (Rabbitts et al. 1985) respectively. However, it is has been shown that the half-life of the message is by no means absolute. For example, Lacy et al. (1989) demonstrated that the half-life of c-*myc* mRNA in an Epstein-Barr virus (EBV)-negative lymphoma cell line was increased from less than 36min. to greater than 70 min. following *in vitro* infection with EBV. However, the significance that these findings have to the regulation of c-*myc in vivo* is yet to be established.

## CHAPTER 2: Phenotypic effects of deregulated c-*myc* expression

### 2.1 The role of c-*myc* in tumourigenesis: co-operativity with other oncogenes.

It has become well established that the complete malignant transformation of any particular cell type can not be achieved through the activation of only one proto-oncogene (Land et al. 1983b; Vogelstein and Kinzler, 1992). As a result, one of the main themes in cancer research has been to elucidate which genes can be considered to be either similar, complementary or synergistic in function during the establishment of a neoplastic phenotype. Indeed, in light of its frequent activation in many tumour types (Yokota et al. 1986; Field and Spandidos 1990), the *myc* oncogenes have been at the centre of many of these studies. For example, as described previously (section 1.1), initial analyses which used the ability to form colonies in semi-solid agar as an *in vitro* assay of cellular transformation, revealed that the c-Ha-*ras* and *raf*-1 genes were able to co-operate with the c-*myc* when co-expressed in rat embryo fibroblasts (Land et al. 1986) and haemopoietic cells (Principato et al. 1990) respectively. However, while these *in vitro* studies have provided great insight into the complementary nature of these oncogenes, the relevance that they have to Myc-associated tumourigenesis *in vivo* has more recently been treated with some caution. In this regard, it was a particularly noteworthy observation that the enforced expression of the c-Myc protein has markedly different consequences, depending on the combination of growth factors in which the cells are maintained (Harrington et al. 1994). In addition, the activity of other oncogenes has been shown to be dependent on both the genetic and mutational background of the cell type used (Newbold and Overell 1983). As a result, to overcome these problems, it was deemed necessary to confirm the effects of an oncogene(s) *in vitro* by assaying for its effects on tumour formation *in vivo*. Although initially this was effectively undertaken by the use of athymic mice, this approach was later

superseded by the generation of animals which carried both widespread and also tissue-specific *myc* transgenes (Adams et al. 1985; Leder at al 1986). The search for co-operating factors in Myc-associated tumourigenesis was then primarily undertaken by either: cross-breeding these transgenic mice with other oncogene-expressing mice, generating mice carrying two oncogenic transgenes, or by infection with oncogenic retrovirus. Outlined below are a number of genes which have been shown to co-operate with the *myc* oncogenes in the establishment of tumours in either transgenic or athymic mice.

### 2.1.1 *Oncogenes which are considered to stimulate cell proliferation.*

#### *1.abl.*

The c-*abl* gene was first discovered as the cellular homologue of the oncogene, v-*abl*, of the Abelson murine leukaemia virus (A-MuLV) (Goff et al. 1980). The role of this gene in the neoplasia of mammals has been highly documented both through its involvement in A-MuLV (Kurzrock et al. 1988) and more importantly as part of the fusion protein (Bcr-abl), which results from the 'Philadelphia' chromosomal translocation in chronic myeloid leukaemia (Daley and Benneriah 1991). The speculation that c-*myc* and *abl* may be co-operating factors in tumour formation came from the analysis of transgenic mice containing the v-*abl* gene under the control of the immunoglobulin enhancer (Eμ-v-*abl*). While these mice were found to be highly predisposed to the formation of plasmacytomas, closer analysis of these tumours revealed that they also frequently contained re-arrangements at the c-*myc* locus (Rosenbaum et al. 1990). To confirm that c-*myc* and *abl* were co-operating in these mice, cross-breeding was undertaken with mice carrying the transgenes, Eμ-v-*abl* and Eμ-c-*myc*. Indeed, the progeny of these mice had a much greater incidence of tumour formation than in either of the parental

strains (Rosenbaum et al. 1990), therefore confirming the synergistic effects of the two genes in the formation of plasmacytomas.

*2. Cyclin D1.*

The first description of the *cyclin* D1 gene came from the analysis of an inversion of chromosome 11 which occurs in a small proportion of benign human parathyroid adenomas (Arnold et al. 1989). This re-arrangement places the *cyclin* D1 gene, which normally resides at chromosome band 11q13, under the control of the regulatory elements of the parathyroid hormone gene on band 11p15 and results in elevated levels of *cyclin* D1 mRNA (Rosenberg et al. 1990). Following these reports it was also found that *cyclin* D1 was also activated as part of a chromosomal translocation, *BCL1* t(11;14), which had been characterised as a consistent feature of certain types of B cell lymphoma (Lammie and Peters, 1991; Raffeld and Jaffe, 1991). In this situation, the regulation of the *cyclin* D1 gene comes under the control of the immunoglobulin enhancer and again results in increased levels of *cyclin* D1 mRNA (Raynaud et al. 1993). In an attempt to generate an animal model of this lymphoma mice were generated which contained an Eμ-*cyclin* D1 transgene (Bodrug et al. 1994). However, it was surprising to find that not only did these mice not develop lymphoma, but their lymphocytes appeared to have the same characteristics as those from non-transgenic animals. In light of this confusing observation, mice were generated which co-expressed the *cyclin* D1 gene with either N-*myc* or L-*myc*. In contrast to the animals which expressed the *cyclin* D1 gene alone, these 'double-transgenics' were found to have a greatly increased rate of lymphomagenesis (Lovec et al. 1994).

In light of the fact that the *cyclin* D1 gene has been shown to be transcriptionally regulated by c-Myc (see section 1.2.2), and that they therefore probably form part of the same signalling pathway in the cell, it is perhaps surprising

that *cyclin* D1 can co-operate with *myc* genes in the establishment of malignant lymphoma. However, it would be naive to assume that the transforming properties of the myc genes are solely determined by the aberrant regulation of *cyclin* D1. For example, as described previously (Packham and Cleveland 1994), the mitogenic properties of c-Myc have also been shown to be dependent on the activity of *ODC*. Alternatively, it might also be the case, that the synergistic effects of the 'double-transgenics' reflects a capacity of N-or L-Myc that is distinct from any of the functions of the c-Myc protein.

### 2.1.2 *Oncogenes which suppress apoptosis.*

#### *1.bcl-2.*

It is now considered that the activation of a proto-oncogene can contribute to cellular transformation, not only through the stimulation of cell-cycle progression, but also through the inhibition of programmed cell death (apoptosis) (Harrington et al. 1994b). One such gene which has become a paradigm for this type of oncogene is *bcl*-2 (Reed 1994). This gene was first isolated from the breakpoint of a chromosomal translocation (t(14;18)), that is a characteristic marker of the majority of non-Hodgkins' B-cell lymphomas (Tsujimoto et al. 1985; Cleary et al. 1986). As a result of this rearrangement the *bcl*-2 gene, which normally resides at chromosome band 18q21, is moved so that it comes under the control of the immunoglobulin heavy chain enhancer (chromosome band 14q32). This has the effect of producing elevated levels of both *bcl*-2 mRNA and Bcl-2 protein (Nowell and Croce 1987). The first indication that *bcl*-2 might contribute to transformation, not through the stimulation of proliferation, but through the inhibition of programmed cell death, was provided in a report by Vaux et al. (1988). They found that the introduction of *bcl*-2 into an interleukin 3 (IL-3)-dependent cell line resulted in prolonged cell survival in the absence of IL3, but without any increase in cell

proliferation. The proof that this effect was indeed as a result of inhibiting programmed cell death was then later provided by Hockenbery et al. (1990).

To investigate the importance of *bcl*-2 in the establishment of lymphoma *in vivo*, transgenic mice were generated which carried the *bcl*-2 gene under the control of the immunoglobulin (Eμ) enhancer. Analysis of these mice revealed that, although they did not develop lymphoma, they had a considerable increase in numbers of resting B cells (McDonnell et al. 1989). In light of the effects of *bcl*-2 *in vitro* (Hockenbery et al. 1990), this finding led to the hypothesis that if this increase in B cell number had also arisen through an inhibition of programmed cell death then these mice would be likely to be highly susceptible to further mutation. Indeed, albeit after a long latency period, these mice went on to develop both lymphomas and plasmacytomas. Moreover, the subsequent analysis of these tumours revealed that 7 out of 15 had a re-arranged c-*myc* gene and an increased abundance of c-*myc* mRNA (McDonnell and Korsmeyer 1991). However, as the activation of c-*myc* may only have been one out of series of mutational events during the formation of these lymphomas, this finding was insufficient evidence to prove a role for c-*myc* in the aetiology of this disease. To clarify this situation, Strasser et al. (1990) generated mice which carried both *bcl*-2 and c-*myc* transgenes. These 'doubly-transgenic' mice developed tumours at a much higher rate than mice carrying either c-*myc* or *bcl*-2 alone and therefore provided a formal indication of the co-operative nature of these two oncogenes.

In light of the conflicting roles of c-Myc in both cell-cycle progression and apoptosis (see section 2.3) it is clear that *bcl*-2 is a highly suitable co-operating factor in Myc-associated tumourigenesis. As *in vitro* studies have shown that *bcl*-2 is effective in blocking c-Myc-mediated apoptosis (Bissonnette et al. 1992; Wagner et al. 1993), this creates a situation in which activation of c-*myc* would cause increased cell-cycle progression without any adverse effects on cell survival. It has now been shown that a number of other proliferation-associated

oncogenes also have the capacity to induce programmed cell death (Debbas and White 1993; Smeyne et al. 1993; Hiebert et al. 1995). It will be interesting therefore, to determine if *bcl*-2 can also co-operate with these factors in processes of transformation both *in vitro* and *in vivo*.

*2. Epstein-Barr virus.*

The pathogenesis of endemic Burkitt's lymphoma (eBL), which is prevalent in Eastern equatorial Africa and New Guinea, is consistently associated with infection with Epstein-Barr virus (EBV) (Epstein and Achong 1983). However, as infection is considerably more widespread than the disease, this indicates that there are other factors, in addition to EBV, that are involved in the establishment of this lymphoma. In this regard, cytogenetic analysis of eBL revealed that the cells of the tumour frequently contained re-arrangements of the c-*myc* gene. In a similar manner to the activation of *bcl*-2 in follicular lymphoma, the c-*myc* gene (chromosome 8) is brought under the control of the enhancer of one of the immunoglobulin loci on chromosomes 2, 14 or 22 (Taub et al. 1982; Adams et al. 1983; Pellici et al. 1986). As it had been shown both *in vitro* (Schwartz et al. 1986) and *in vivo* (Langdon et al. 1986) that the deregulated expression of c-*myc* in B cells was insufficient to induce lymphoma, it was speculated that there may be a co-operative effect between c-*myc* and EBV. To test this, Lombardi et al. (1987) introduced a c-*myc* gene under the control of the SV40 enhancer into human non-BL-derived, EBV-infected, B cells. Indeed, this resulted in the tumourigenic conversion of these cells as assessed by an ability to form tumours in athymic mice. As no tumours were formed when cells were injected which contained either EBV or c-*myc* alone, this gave a clear indication of the co-operative nature of the two agents.

A possible mechanism as to how EBV might co-operate with c-*myc* in tumourigenesis was later provided by Gregory et al. (1991). This study reported that the expression of all eight EBV 'latent' proteins resulted in an inhibition of B-cell apoptosis. Moreover, it was later shown that this effect was solely attributable to one of these proteins, latent membrane protein 1 (LMP 1), and that this protein mediates this effect through the transactivation of *bcl*-2 (Henderson et al. 1991). Indeed, this scenario is therefore very similar to the co-operativity between c-*myc* and *bcl*-2 that has been described during the formation of follicular lymphoma (see above). However, there is one caveat. Analysis of the expression of 'latent' proteins in samples of established eBL revealed that the only protein expressed was not LMP-1, but EBNA-1 (Rowe et al. 1987; Rogers et al. 1992). This might therefore indicate that the suppression of apoptosis by LMP-1 may only function to render B cells susceptible to mutations which result in the formation and maintenance of a phenotype in which LMP-1 is not required. It remains to be seen if this is also a facet of other viruses that have also been implicated in neoplasia, but are largely found associated with non-pathological latent infection, for example, HPV (Pfister 1992; Gissmann 1992).

### 2.1.3 *The search for novel factors which can co-operate with myc genes in tumourigenesis.*

It is well established that the formation of lymphoid tumours in Eμ-*myc* transgenic mice only occurs after a long latency period (Harris et al. 1988; Schmidt et al. 1988). Since analysis of these tumours revealed that they were clonal and did not contain alterations of the transgene, it was postulated that they had most likely arisen through the mutation of other genes which co-operate with c-*myc* in the establishment of the disease (Alexander et al. 1987; Webb et al. 1989). However, although this hypothesis was perfectly valid, the identity of these genes was

completely unknown. In an attempt to identify these co-operating factors, the Eμ-*myc* mice were subjected to retroviral-insertional mutagenesis using the Moloney murine leukaemia virus (Mo-MuLV). The rationale for this approach was based on the fact that the random integration of Mo-MuLV results in the deregulation or inactivation of loci throughout the genome. When integration occurs at a gene which can co-operate with c-*myc* this may well lead to the formation of tumours. The sequences of the virus can then be used as a 'tag' to facilitate the identification of the novel factors involved (for reviews see: Peters 1990; van Lohuizen and Berns 1990). As a result of this approach, five loci: *pim*-1, *bmi*-1, *emi*-1, *pal*-1 and *bla*-1, were frequently found at integration sites of Mo-MuLV in Myc-associated lymphoma (Haupt et al. 1991; van Lohuizen et al. 1991). Although four of these loci had not previously been identified, the *pim*-1 gene had been reported to be the common site of integration in non-*myc*-associated, murine leukaemia virus-induced T-cell lymphoma (Cuypers et al. 1984; Selten et al. 1985). As a result, much of the follow-up work on these loci has concerned this gene. In particular, in order to prove the co-operative nature of c-*myc* and *pim*-1 in lymphomagenesis, Verbeek et al. (1991) generated mice that contained both Eμ-*myc* and Eμ-*pim*-1 transgenes. Analysis of these mice revealed that they develop a prenatal pre-B cell lymphoma that was not evident when either gene was expressed individually.

To investigate the functions of *pim*-1 *per se*, Laird et al (1995) inactivated the gene by homologous recombination. As these mice show only subtle phenotypic alterations, it was postulated that there may be another gene which can complement for a deficiency in *pim*-1. Based on these thoughts, Eμ-*myc*/*pim*$^{-/-}$ mice were infected with Mo-MuLV in an attempt to identify other factors which can associate with c-*myc* in tumourigenesis. This led to the identification of a novel gene, *pim*-2, which was found to be 53% identical to *pim*-1 at the amino acid level (van der Lugt et al. 1995).

Indeed, while the discovery of these novel factors is extremely interesting, at present very little is known about their function and the way in which they can contribute to tumourigenesis. More work is therefore required not only to elucidate their role with respect to c-Myc, but also to determine if these factors function as oncogenes in the establishment of cancer in humans.

## 2.2 Distinct roles of the two forms of the c-Myc protein in cell growth and tumourigenesis.

A distinctive feature of the c-*myc* gene is that it encodes two alternatively translated protein products (Hann and Eisenman 1984; Ramsey et al. 1986; Dosaka-Akita et al. 1991)(Figure 1). The proteins differ not only in size, but also in the mechanism by which their translation is initiated (Hann et al. 1988). The shorter and more predominant form of c-Myc, c-Myc 2, is initiated from a standard AUG site, whereas the longer protein, c-Myc 1, is initiated from a non-AUG site (CUG in humans) (Hann et al. 1988). As it was noticed that c-Myc 1 was induced to levels comparable to those for c-Myc 2 as cells reached high densities during *in vitro* culture (Hann et al. 1992), it was considered that the two forms of the Myc protein may perform different roles. In order to investigate this Hann et al. (1994) generated clones of COS cells which expressed high levels of either c-Myc 1 or c-Myc 2. These cells were then transiently transfected with different regions of the Rous sarcoma virus long terminal repeat, to assess the possibility that the two Myc proteins possess distinct transcriptional activities (Hann et al. 1994). The study revealed that one specific enhancer element, EFII, was activated by c-Myc 1, but not by c-Myc 2 (Figure 5). Surprisingly, when the sequence of EFII was analysed, it was noticed that it did not contain the consensus Myc:Max binding site (CACGTG) and that the activation by c-Myc 1 was dependent on a repeat sequence that contained a consensus binding site for a CCAAT/enhancer binding

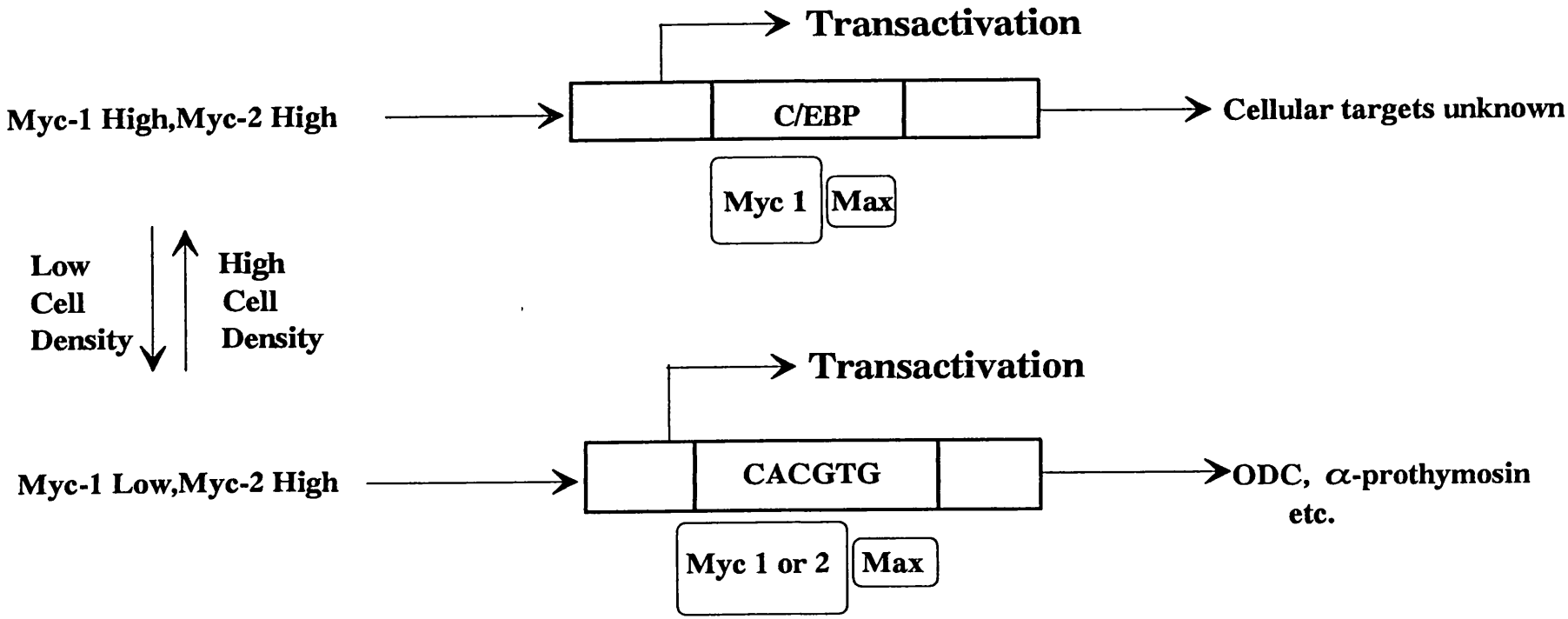



Figure 5: *Implications of the different properties of the two Myc proteins, c-Myc 1 and c-Myc 2.*

C/EBP, CCAAT/enhancer binding protein; ODC, ornithine decarboxylase.

protein (C/EBP). Subsequent EMSA of this binding site showed, however, that it too was bound by c-Myc 1:Max heterodimers, but not by Max:Max homodimers. This therefore opens up the possibility of a situation in which Myc:Max and C/EBP are in competition for the same binding site. However, the consequences that this might have *in vivo* are still unknown.

Although any cellular targets for transcriptional regulation by c-Myc 1 are yet to be discovered, the implications of this protein are extremely interesting since COS cells in which it is over-expressed display a growth-inhibited phenotype (Hann et al. 1994). It is also noteworthy that in cases of Burkitt's lymphoma in which the c-*myc* gene is translocated to the immunoglobulin locus the re-arrangement frequently occurs such that only c-Myc 2 becomes activated (Dalla-Favera et al. 1983; Rabbitts et al. 1983). Also, in cases in which the translocation results in both c-Myc 1 and c-Myc 2 activation, it has been reported that the c-Myc 1 protein is frequently mutated (Rabbitts et al. 1984). In addition, in all cases of Burkitt's lymphoma that display this reciprocal translocation, the non-translocated c-*myc* allele has been reported to be silenced (Bernard et al. 1983). In this regard, it is now perhaps the time to re-investigate whether the c-*myc* gene is mutated in other tumours such that only c-Myc 2 becomes activated.

### 2.3 The involvement of c-Myc in programmed cell death (apoptosis).

Undoubtedly, the most paradoxical revelation from the recent work on c-Myc was the finding that under certain circumstances the protein is able to induce programmed cell death (apoptosis). Two separate studies, one in fibroblasts (Evan et al. 1992) and one in haemopoietic cells (Askew et al. 1991), revealed that when Myc was constitutively expressed the cells required the presence of exogenous growth/survival factors in order to replicate effectively. If serum in the case of fibroblasts, or interleukin 3 in the case of haemopoietic cells, was withdrawn from

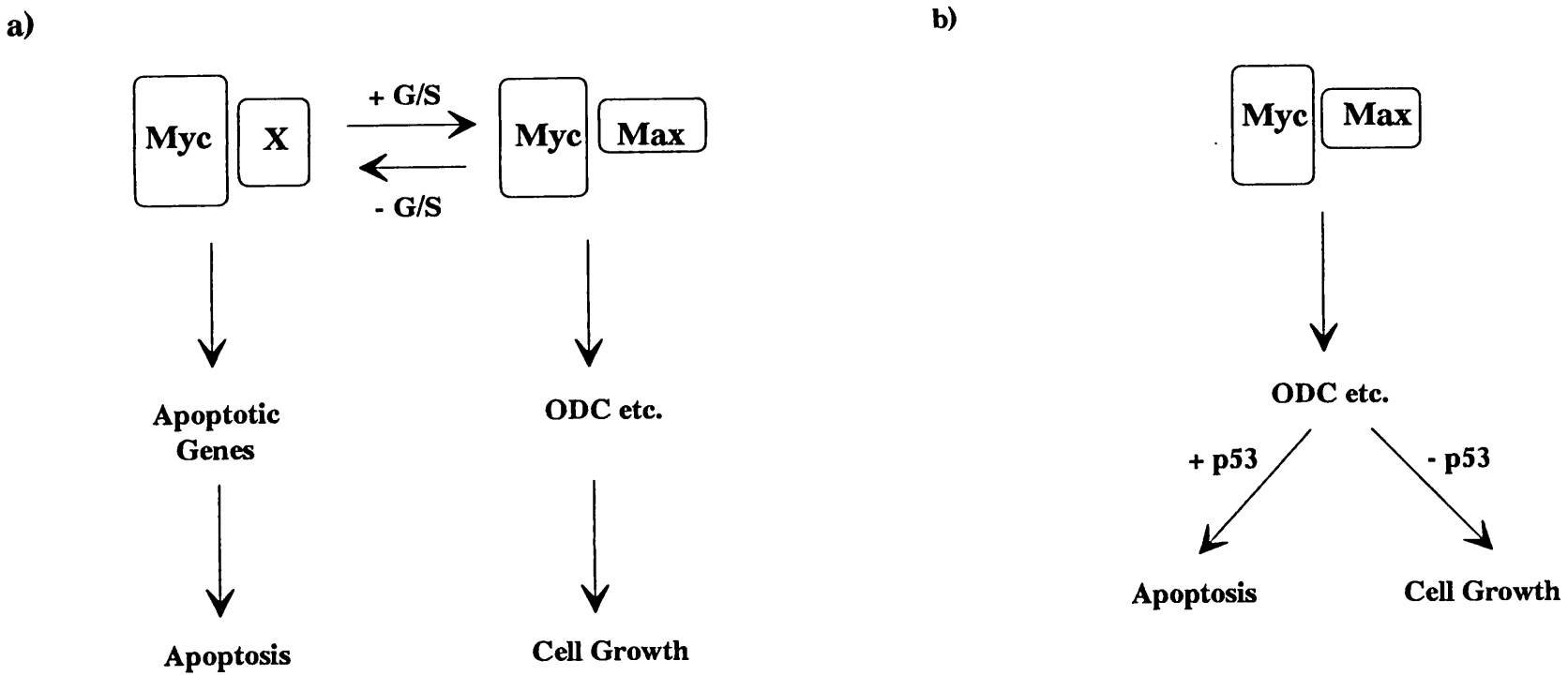



Figure 6: *Possible mechanisms of Myc-induced apoptosis.*

a) A pathway involving two sets of Myc-regulated genes. b) A pathway involving one set of Myc-regulated genes, in which the decision between cell growth and cell death is mediated by p53. G/S, growth/survival factors.

the Myc-expressing cultures the cells continued to enter the cell cycle, but then underwent apoptosis. Two possible explanations for how the cell might make a choice between life and death as a result of Myc expression are outlined in Figure 6. Initially it was thought possible that the absence of exogenous growth/survival factors was having an effect upon the transcriptional targets of c-Myc (Figure 6a). As described earlier (section 1.3) this could perhaps occur through a change in the relative abundances of transcriptional complexes involving the Myc protein, for example, a change from a predominance of Myc:Max complexes to a predominance of Myc:TFII-I complexes. However, since it has now been shown that the apoptotic cell death induced by Myc requires dimerization with Max (Amati et al. 1993b) and is dependent on an active ODC protein (Packham and Cleveland 1994), it seems more likely that the same genes are transcribed in both the absence and presence of growth/survival factors and that the decision between life and death is made elsewhere (Figure 6b). The observation that the ability of Myc to induce apoptosis is abrogated in cells lacking a wild-type p53 protein (Hermeking and Eick 1994) points to an involvement of p53 at some point during the apoptotic programme. In this regard it is interesting to note that apoptosis induced by the adenovirus E1A protein is also mediated by p53 (Debbas and White 1993). However, whether the p53 protein acts through this mechanism to eradicate cells which contain an aberrant growth stimulus induced by other oncogenes is yet to be established.

## 2.4 The role of c-Myc in the control of differentiation.

The first indication that c-*myc* might be involved in the control of cellular differentiation came from the observation that the abundance of c-*myc* mRNA is markedly decreased during the terminal differentiation of Friend-murine erythroleukaemia (MEL) cells and murine myeloid leukaemia cells (Gonda and Metcalf 1984; Lachman and Skoultchi 1984). This effect on c-*myc* mRNA was then

later also reported to occur during the differentiation of a number of other cell types including, F9 teratocarcinoma cells and HL60 promyelocytic leukaemia cells (Griep and DeLuca 1986; McCachren et al. 1986). This causal association between c-*myc* and differentiation was then subsequently confirmed in one of two ways. Firstly, the constitutive expression of c-*myc* in MEL cells was found to inhibit the differentiation programme which normally occurs following treatment with DMSO or hypoxanthine (Coppola and Cole 1986; Dmitrovsky et al. 1986; Prochownik and Kukowska 1986). In addition, the introduction of constructs expressing c-*myc* antisense RNA into F9 cells and the treatment of HL60 cells with antisense oligonucleotides directed against the 5' end of the c-*myc* mRNA, was found to cause a programme of differentiation to parietal endoderm and granulocytes, respectively (Griep and Westphal 1988; Holt et al. 1988). However, although it has now also been shown that the ectopic expression of c-*myc* can block both adipogenic and myogenic differentiation (Freytag 1988; Miner and Wold 1988), the significance of c-*myc*'s effects on differentiation with respect to its role in tumourigenesis are as yet unknown. In addition, the mechanism by which c-Myc negatively regulates differentiation also remains elusive. In this regard, one school of thought has grown to consider that c-*myc* indirectly regulates differentiation through its ability to direct cell-cycle progression in cells which must growth arrest in order to differentiate. However, as there is little evidence to support this hypothesis, it is equally possible that c-*myc* controls differentiation by a mechanism that is independent of its effects on cell cycle progression (Figure 7). Outlined below are a number of studies which have attempted to shed light on these interesting and important questions.

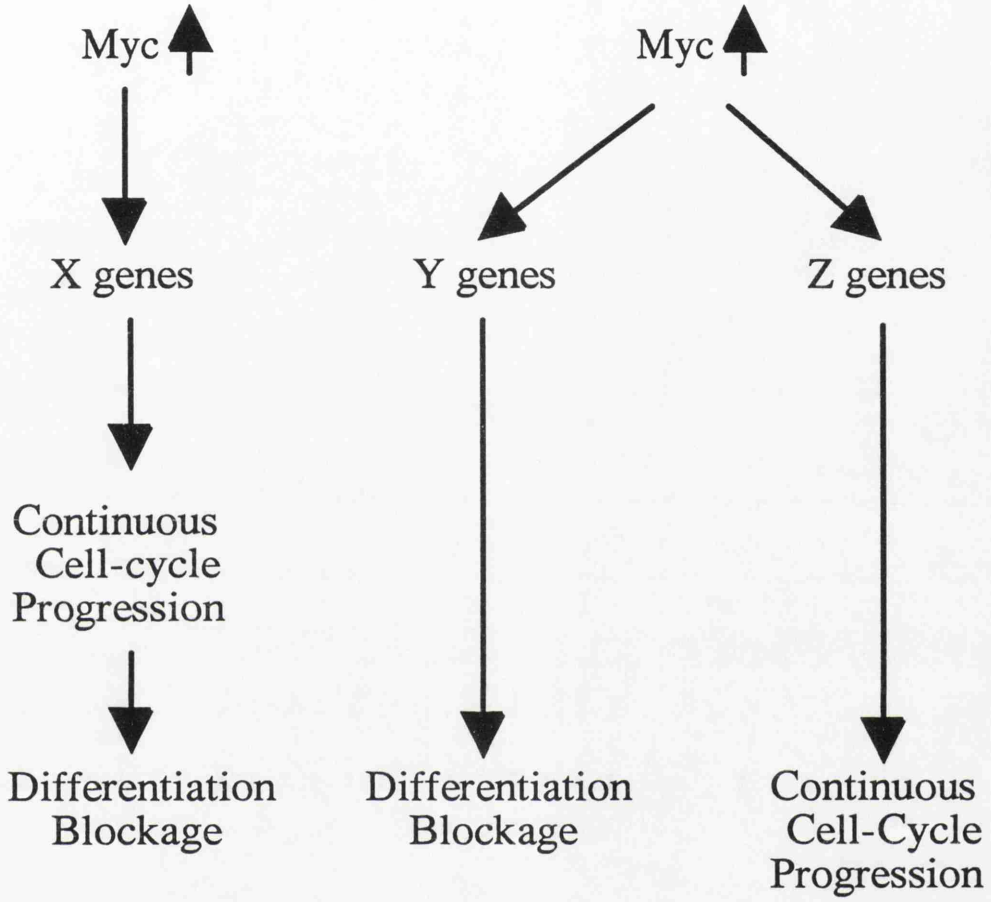



Figure 7: *Representation of the possible mechanisms of Myc-induced differentiation blockage.*

The mechanism on the left involves one set of genes (X) which regulate cell proliferation and as a result indirectly affect differentiation. Alternatively, on the right a model is proposed in which proliferation and differentiation are regulated by two distinct sub-sets of genes, Y and Z. See text for details.

### 2.4.1 *The significance of the capacity of c-myc to negatively regulate differentiation to its ability to co-operate in cellular transformation.*

In order to define which regions of the c-Myc protein are involved its ability to induce cellular transformation, Stone et al. (1987) generated variants of c-Myc which contained either interstitial deletions or point mutations. The genes for these mutants were then transfected, in conjunction with a mutant *ras* gene, into rat embryo cells and, as an assessment of transformed phenotype were then tested for their ability to grow in semi-solid agar. This revealed that there are two regions of the protein that are crucial for transforming capacity (amino acids 105-143 and 321-439), one region which could tolerate small deletions (amino acids 1-104) and one region which was largely dispensable (amino acids 144-320).

On the basis of these findings, it was considered that any of the phenotypic characteristics of c-Myc which are important to its transforming potential would also require the same portions of the protein as described above. To this end, Freytag et al. (1990) and Bar-Ner et al. (1992) analysed these mutants for their ability to block the differentiation of 3T3-L1 adipocytes and MEL cells respectively. This revealed that, in both systems, the regions of the protein that are essential for transformation are also fundamental for its ability to block differentiation. This therefore indicates that the role of c-Myc in the control of this process may well be related to its function in the establishment of neoplastic disease.

### 2.4.2 *Possible mechanisms by which c-myc negatively regulates cellular differentiation.*

Support for the hypothesis that c-*myc* blocks differentiation as an indirect effect of its control of the cell cycle has been presented by Freytag (1988). In this study, 3T3-L1 preadipocytes were transfected with a c-*myc* gene under the

control of the Rous sarcoma virus promoter. These cells were then analysed for their ability to undergo three important steps in the establishment of a terminally differentiated adipoblast: a) the ability to growth arrest in $G_0/G_1$ as the cells reach confluence prior to differentiation induction, b) the ability to replicate the genome following treatment with the differentiation inducers, c) loss of responsiveness to mitogens after 48hr of differentiation induction. Surprisingly, it was found that the c-*myc*-expressing cells were indistinguishable from the non-transfected cells when analysed for criteria a and b, but were different in that, after 48hr of treatment with the differentiation inducers, they re-entered the cell cycle and failed to differentiate when they were treated with 30% serum. These findings led Freytag to conclude that it was not, as had previously been considered, through the stimulation of cell-cycle progression that c-Myc prevents terminal differentiation. Instead, it was proposed that the continued expression of c-Myc precludes an irreversible exit from the cell cycle that is clearly a prerequisite for differentiation in these cells. In line with a previous study (Scott et al. 1982), it was formulated that this growth arrest, through its irreversible nature, was different from that associated with quiescence and to distinguish between the two, they were termed $G_D$ and $G_S$, respectively.

Indeed, although these findings are extremely interesting, it must be pointed out that the culture conditions used for growth of 3T3-L1 are markedly different from those in which the cells are differentiated. In this regard, as it has been shown that the activity of c-Myc is highly dependent on external stimuli (Evan et al. 1992; Harrington et al. 1994), these findings may well indicate functions of c-Myc that are not involved in adipogenesis *in vivo*. It will therefore be interesting to discover if the conclusions drawn by Freytag (1988) are also applicable to other lineages where withdrawal from the cell cycle is considered a pre-prerequisite for terminal differentiation.

## CHAPTER 3: *In vitro* models of myeloid differentiation used in this study.

### 3.1 The HL60 cell line.

The HL60 cell line was derived from a patient with myeloid leukaemia (Collins et al. 1977). Although it was initially considered that the classification of this leukaemia was promyelocytic (FAB Class M3), it was later re-evaluated as acute myeloblastic leukaemia with differentiation (FAB Class M2) (Dalton et al. 1988). Most notably, this re-evaluation discovered that HL60 cells do not contain the t(15;17) translocation that is a characteristic marker of FAB Class M3 (Dalton et al. 1988). The subsequent characterisation of HL60 cells revealed that they can continuously proliferate in suspension culture and, depending on sub-line, have a doubling time of ~36 to 48hr (Gallagher et al. 1979). In addition, attempts to grow the line in serum-free conditions revealed that its growth was not only dependent on exogenous insulin and transferrin (Breitman et al. 1980), but also on the presence of an autocrine growth factor which is continuously produced by the cells (Brennan et al. 1981).

#### 3.1.1 *Induction of differentiation of HL60 cells.*

When maintained in suspension culture, the majority of HL60 cells (~95%) exhibit characteristics of cells arrested at the promyelocytic stage of differentiation. The remaining 5% represent cells which, through spontaneous differentiation, have progressed past the promyelocytic stage and exhibit characteristics of more mature cells, including myelocytes, metamyelocytes and neutrophils (Gallagher et al. 1979). Undoubtedly, the most interesting feature of HL60 cells is that the percentage of cells which exhibit a more mature phenotype can be enhanced by treatment with a variety of physiological and chemical inducers

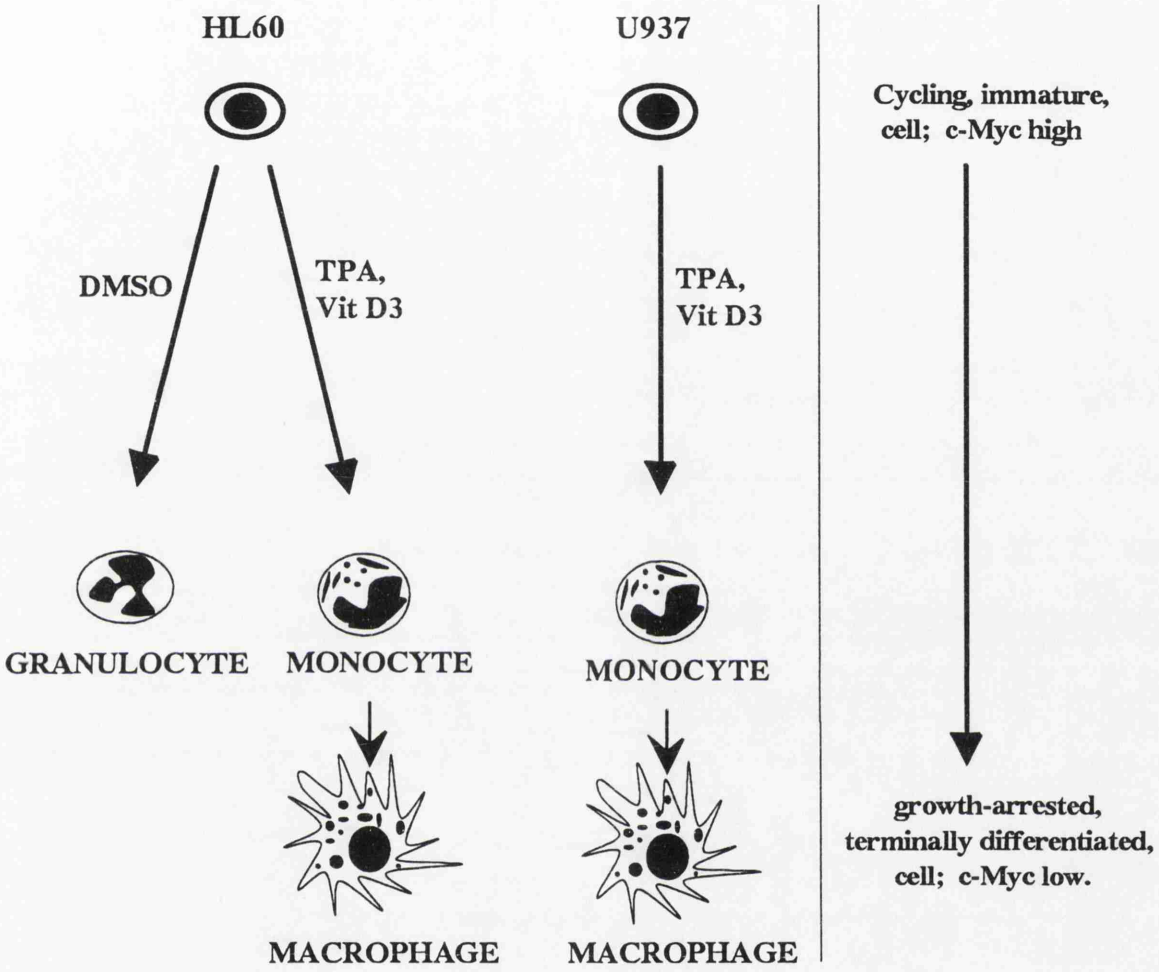



Figure 8: *Schematic representation of the leukaemic cell lines used in this study.*

Abbreviations used: DMSO, dimethyl sulphoxide; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; Vit D3, 1,-α,25-dihydroxyvitamin D3. See text for details.

(Table 3). Moreover, depending on the inducer used, the cells can be stimulated to differentiate independently down two pathways to either granulocytes or monocytes/macrophages (Figure 8) (Tsiftsoglou and Robinson 1985). Analysis of these cells for terminal differentiation markers following differentiation induction routinely reveals that in excess of 80% of the cells acquire a mature phenotype. Also, concomitant with this differentiation, the cells undergo a growth arrest in the $G_0/G_1$ phase of the cells cycle, which is then ultimately followed by a programme of cell death (Mitchell et al. 1992).

As many of the processes that occur during the induced differentiation of HL60 cells develop simultaneously with many other events, analysis of any particular phenotypic change is very difficult. As a result, a number of HL60 variants have also been isolated which differ in their response to treatment with the differentiation inducer (Toksoz et al. 1982; Bunce et al. 1983; Anderson et al. 1985).

### 3.1.2 *Oncogenes and tumour suppressor genes in HL60 cells.*

#### *1. c-myc.*

The first oncogene to be found altered in HL60 cells was c-*myc*. Two reports which analysed the DNA from these cells indicated that the gene was amplified approximately 30-fold (Collins and Groudine 1982; Dalla-Favera et al. 1982). Moreover, the subsequent analysis of the original tumour material revealed that this amplification had arisen during the establishment of the disease and not from the culture of the cells *in vitro*. This discovery was in fact, the first report of the amplification of a cellular oncogene in human cancer. However, as the result of a survey of more than 100 leukaemias by Rothberg et al. (1984), it would appear that amplification of c-*myc* is not a common event in haemopoietic neoplasia, with only one of the samples examined exhibiting this change. It is also clear that the

| Differentiation Inducer | Induction of HL60 cells to granulocytes | Induction of HL60 cells to monocytes/ macrophages | Induction of U937 cells to monocytes/ macrophages |
|---|---|---|---|
| TPA | | * | * |
| DMSO | * | | * |
| IFN $\gamma$ | | * | * |
| Retinoic Acid | * | | |
| Lymphocyte conditioned media | | * | * |
| 1-$\alpha$,25-dihydroxy-vitamin D3 | | * | * |
| Teleocidin | | * | |
| Actinomycin D | * | | |

Table 3: Factors known to induce differentiation in HL60 and U937 cells.

Abstracted from Collins (1987) and Harris and Ralph (1985).

Abbreviations used: TPA, 12-0-tetradecanoyl-phobol-13-acetate; DMSO, dimethyl sulphoxide; IFN$\gamma$, interferon gamma.

high level of c-*myc* amplification in HL60 cells (30-fold) is not an essential change for the continued survival of the cell line, as a number of sub-lines have been reported which contain amplifications as low as only 4-fold (Graham et al. 1985; Donti et al. 1991). However, the consistent finding that expression of c-*myc* is incompatible with terminal differentiation (see section 2.4), lends heavy support to an involvement of *myc* amplification, even when only 4-fold, in the maturation-arrested phenotype of HL60 cells.

*2. N-ras.*

In a study by Murrey et al. (1983), it was found that the transfer of HL60 genomic DNA into NIH3T3 cells resulted in their transformation. However, it was discovered that the gene responsible for this effect was surprisingly not c-*myc*, but N-*ras* (Murrey et al. 1983). In addition, in contrast to the situation in other myeloid leukaemias where the N-*ras* gene is frequently mutated at codons 12 and 13 (Bos et al. 1985; Needleman et al. 1986), in HL60 cells the gene is mutated at codon 61 (Bos et al. 1984). However, as little is yet known about the physiological function of N-*ras*, more work is now required to establish the role it plays in both the establishment of myeloid leukaemia and the phenotype of HL60 cells.

*3. p53*

In light of the fact the p53 gene is the most frequently mutated gene in human cancer (Hollstein et al. 1991), it is perhaps no surprise that HL60 cells also carry mutations at this locus. In a report by Wolf and Rotter (1985), it was found that the p53 gene, which resides on the short arm of chromosome 17, is largely deleted in HL60 cells. In addition, the remaining allele of p53 is also deleted as part

of the monosomy of chromosome 17 that was identified during the initial characterisation of the line (Gallagher et al. 1979). As a result of these changes the cells have been shown to be devoid of both p53 mRNA and protein (Wolf and Rotter 1985). However, it not known how this loss of p53 relates to the establishment of the original leukaemia or to the maturation-arrested phenotype of HL60 cells.

### 3.2 The U937 cell line.

The U937 cell line was originally derived from a patient with diffuse histiocytic lymphoma (Sundstrom and Nilsson 1976). The line is similar to the HL60 cell line in that it can be maintained indefinitely in suspension as a population of blast cells of the myelomonocytic lineage (Harris and Ralph 1985). However, in contrast to the bipotential nature of HL60 cells, the U937 cell line can only be stimulated to differentiate to monocytes/macrophages (Figure 8; Table 3). As a result, U937 cells have not received as much attention as HL60 cells and remain less well defined. In this regard, there have been no reports relating to any mutational events in the cells which may have given rise to the original disease. However, it has been shown that, the constitutive expression of c-myc in U937 cells results in a block to their ability to differentiate in response to treatment with TPA (Larsson et al. 1988).

**Initial aims of the project.**

It had been known for some time that constitutive expression of c-*myc* can block the differentiation of myeloid cells. However, it is also now evident that the correct functioning of c-Myc is not only dependent on the abundance of its dimerization partner Max, but also on the levels of two other Max-binding proteins, Mad and Mxi1. In light of this, it was the initial aim of this project to investigate further the role of c-Myc in the regulation of myeloid differentiation, and with particular interest being given to any involvement that Max and then subsequently Mad and Mxi1 may have in the control of this process.

# MATERIALS AND METHODS

# CHAPTER 4: MATERIALS

## 4.1 Tissue culture media and supplies.

Supplier: *Beatson Institute Central Services.*

Sterile $dH_2O$

Penicillin (7.5 mg/ml).

Streptomycin (10 mg/ml).

Sterile PBS-EDTA.

Sterile PBS.

Sterile glassware and pipettes.

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

Special liquid medium (SLM).

200mM glutamine.

100mM sodium pyruvate.

7.5% (w/v) sodium bicarbonate.

2.5% (w/v) trypsin.

Foetal calf serum.

Supplier: *A/S Nunc, Roskilde, Denmark.*

Tissue culture flasks.

Nunc cryotubes.

Chamber microscope slides.

Supplier: *Fisons Scientific Equipment, Loughborough, Leicestershire, England.*

dimethyl sulphoxide (DMSO).

Supplier: *Biological Industries, Kibbutz Beth, Haemek, Israel.*

10x RPMI medium.

### 4.2 Plasticware.

Supplier: *Becton Dickinson Labware, Plymouth, England.*

Falcon tubes.

Supplier: *Bibby-Sterilin Ltd., Stone, Staffordshire, England.*

30ml universal and 5ml bijou tubes.

Supplier: *BDH Chemicals Ltd., Poole, Dorset, England.*

Glass microscope slides.

### 4.3 Bacterial hosts and media.

Supplier: *Beatson Institute Central Services.*

L-broth, prepared as outlined in Sambrook et al. (1989).

Sterile glassware.

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

DH5α competent cells.

Supplier: *Difco, Detroit, Michigan, USA.*

Bacto-agar.

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

Ampicillin.

Tetracycline.

Supplier: *Bibby-Sterilin Ltd., Stone, Staffordshire, England.*

Petri dishes.

### 4.4 Nucleotides, Polynucleotides and DNA.

Supplier: *Amersham International plc. Amersham, Buckinghamshire, England.*

[α-$^{32}$P] dCTP ~3000Ci/mmol.

[γ-$^{32}$P] ATP >5000Ci/mmol.

[α-$^{35}$S] dATP >1000Ci/mmol.

Unlabelled nucleotides.

Supplier: *Pharmacia Ltd., Milton Keynes, Buckinghamshire, England.*

Poly [dI-dC]•poly[dI-dC].

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

Salmon sperm DNA.

**4.5 Chemicals.**

All chemicals not individually listed were obtained (AnalaR grade) from BDH Chemicals Ltd., Poole, Dorset, England.

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

| | |
|---|---|
| Bromophenol blue. | Ethidium bromide. |
| Dithiothreitol. | MOPS. |
| Polyvinylpyrrolidone. | Ficoll. |
| Bovine serum albumin (Fraction V). | TEMED. |
| Tween 20. | TPA. |
| NBT. | NP40. |
| Spermidine. | Chromomycin A3. |
| OAG. | Ponceau S. |
| PMSF. | Putrescine. |

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

Ethanol.

Supplier: *Rathburn Chemicals Ltd., Walkerburn, Scotland.*

Water-saturated phenol.

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

RNAzol B.

Supplier: *Fisons Scientific Equipment, Loughborough, Leicestershire, England.*

38% (w/v) Formaldehyde.

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

Formamide.

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

CsCl.

HEPES.

Supplier: *Bethesda Research Laboratories, Life Technologies, Inc., USA.*

Agarose, ultrapure electrophoresis grade.

Supplier: *National Diagnostics, Manville, New Jersey, USA.*

Ecoscint A.

Supplier: *Calbiochem-Novabiochem (UK) Ltd., Beeston, Nottinghamshire, England.*

1-α,25-dihydroxyvitamin D3

Supplier: *Biorad Laboratories Ltd., Hemel Hempstead, Hertfordshire, England.*

Bradford protein assay reagent.

### 4.6 Antisera

Supplier: *Santa Cruz Biotechnology Inc., Santa Cruz, California, USA.*

Max1 antisera: Rabbit IgG polyclonal (C-17).

Mad antisera: Rabbit IgG polyclonal (C-19).

Supplier: *Promega Ltd., Southampton, Hampshire, England.*

Goat anti-rabbit IgG horseradish peroxidase conjugate.

The sources of antisera obtained as gifts from other academic laboratories are listed where quoted.

### 4.7 Enzymes and enzyme inhibitors.

All DNA modifying enzymes and their buffers, except those listed below, were obtained from: *Bethesda Research Laboratories, Gibco Ltd. Paisley, Scotland.*

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

Klenow Polymerase

Proteinase K

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

AMV reverse transcriptase.

RNAse A.

T4 polynucleotide kinase.

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

Diethylpyrocarbonate (DEPC).

Lysozyme.

**4.8 Gels, binding matrices and Columns.**

Supplier: *Severn Biotech Ltd., Kidderminster, England.*

Design-a-Gel 29% (w/v) acrylamide, 1% (w/v) bisacrylamide. solution.

Design-a-Gel 29.6% (w/v) acrylamide, 0.4% (w/v) bisacrylamide. solution.

Supplier: *Pharmacia Ltd., Milton Keynes, Buckinghamshire, England.*

Nick columns.

Supplier: *Dynal A.S, Oslo, Norway.*

M-280 Streptavidin-labelled Dynabeads.

**4.9 Kits.**

Supplier: *Amersham International plc, Amersham, Buckinghamshire, England.*

ECL western blotting detection kit.

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

DNA random priming labelling kit.

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

Kit 91-A: α-napthyl acetate esterase detection kit.

Supplier: *Bio 101, Stratatech Scientific, Luton, Bedfordshire, England.*

Geneclean II kit.

Supplier: *United States Biochemical (USB), Cleveland, Ohio, USA.*

Sequenase Version 2.0 kit.

Supplier: *Perkin Elmer Cetus, Norwalk, Connecticut, USA.*

Geneamp DNA PCR kit.

Automated sequencer 'Dyedeoxy' sequencing kit.

**4.10 Membranes, Paper and X-ray film.**

Supplier: *Amersham International plc, Amersham, Buckinghamshire, England.*

Hybond N+ nylon membranes.

Supplier: *Biorad Laboratories Ltd., Hemel Hempstead, Hertfordshire, England.*

Trans-Blot nitrocellulose membranes.

Supplier: *Vernon-Carus Ltd., Preston Lancashire, England.*

Gauze swabs.

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

3MM filter paper.

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

X-ray film (X-OMAT AR).

Duplicating film (DUPC-1).

Duplicating film (DUP-1).

Supplier: *Presentation Technology Ltd., Clydebank, Scotland.*

AGFA Rapitone paper (P1-2, P1-3 and P1-4).

### 4.11 Markers:

Supplier: *Amersham International plc, Amersham, Buckinghamshire, England.*

Prestained 'rainbow' protein markers

range 2,350-46,000Da and range 14 300-220,000Da.

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

ϕX174 RF DNA / Hae III fragments.

λ DNA / Hind III fragments.

RNA ladder.

### 4.12 Water.

De-ionised water ($dH_2O$) for general use and for the preparation of solutions and buffers was obtained from a Millipore MilliRO 15 system. Water for enzymic reactions and recombinant DNA work ($RQH_2O$) was further purified by reverse osmosis on a Millipore MilliQ system to 18Ωcm.

# CHAPTER 5: METHODS

## 5.1 Cell Culture and induction of differentiation.

### 5.1.1 *Culture of HL60, HL60-Ast4, HeLaS3 and HFF cells.*

Passage 25 HL60 cells were kindly provided by Dr. G. Brown (Dept. of Immunology, University of Birmingham, UK). HeLa S3 cells were obtained from Beatson Institute stocks. Both cell lines were grown in suspension culture in Special Liquid Medium, supplemented with 10% foetal calf serum and 2mM glutamine in an atmosphere of 5% $CO_2$ in air at 37°C. Cultures were seeded at a density of 5 x $10^5$ cells/ml and were passaged every 48hr. Differentiation-defective HL60-Ast4 cells were also obtained from Dr. G. Brown and were maintained in the same manner as the parental HL60 cells, except there growth medium was supplemented with 1.25% Dimethyl sulphoxide. Human Foreskin Fibroblast cells were a kind gift from Dr. Ken Parkinson of the Beatson Institute and were grown under the same conditions as those for HL60 and HeLa cells, except the growth medium was supplemented with 15% serum instead of 10%. Confluent cultures of these cells were passaged with a maximum of a 1:4 split.

### 5.1.2 *Culture of U937 cells.*

U937d+ cells were obtained from The European Collection of Animal Cell Cultures, Porton Down, U.K.. U937d- cells were from stocks within the Beatson Institute. Both U937 cell lines were grown in suspension culture in RPMI 1640 medium, supplemented with 10% foetal calf serum, 1mM sodium pyruvate and 2mM glutamine in an atmosphere of 5% $CO_2$ in air at 37°C. These cultures were also passaged every 48hr and were seeded at a density of 5 x $10^5$ cells/ml.

*5.1.3 Cell Storage.*

All of the cell lines used in this study, except HFF cells which were obtained from Dr. Parkinson as and when required, were stored frozen in liquid nitrogen. Cultures of growing cells were harvested by centrifugation at 1,100 rpm for 5min in a MSE benchtop centrifuge. The cell pellets were then resuspended to a final concentration of $10^7$ cells/ml in a solution of the appropriate growth medium containing DMSO at a concentration of 10% (v/v). They were then aliquotted into 1-2ml cryotubes and placed in a polystyrene container at -70°C for 24hr. After this time the vials were placed in liquid nitrogen for long-term storage. Cultures were recovered by transferring the cryotubes directly from the liquid nitrogen into water at 37°C. Once thawed the cells were diluted in 20ml of growth medium, pre-warmed to 37°C, and centrifuged for 5min at 1,200rpm in a MSE benchtop centrifuge. Cells from one vial were then seeded into 5ml of fresh, pre-warmed growth medium.

5.1.4 *Induction of differentiation.*

Prior to induction of differentiation and/or growth arrest, all sublines of HL60 and U937 cells were diluted with fresh medium to a concentration of $5x10^5$ cells/ml, 16hr prior to induction. Growth arrest and/or differentiation of these cells to monocytes/macrophages was achieved by addition of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to a final concentration of $1.6 \times 10^{-7}$M. In addition, "wild-type" HL60 cells were also induced to differentiate with 1-α,25-dihydroxyvitamin D3 to a final concentration of $1 \times 10^{-7}$M, to yield populations of monocytes/macrophages, and with DMSO to 1.5% (v/v) to yield granulocytes.

5.1.5 *Markers of differentiation.*

a) Non-specific esterase (NSE): Aliquots of 1 x $10^6$ cells were collected on glass slides by centrifugation (500rpm for 5 minutes) in a Cyto-tek cytocentrifuge (Miles scientific). Non-specific esterase activity was assessed using diagnostic kit 91-A (Sigma) and assays were performed as recommended by the manufacturer. Two slides were prepared for each time point and 100 cells were examined to determine the proportion staining positively for NSE.

b) Nitroblue tetrazolium (NBT) reduction: Aliquots of 1 x $10^6$ cells were harvested by centrifugation at 1200rpm for 5 minutes (MSE benchtop). The cell pellet was re-suspended in 1 ml of phosphate-buffered saline containing 10μM TPA and 0.2% (w/v) NBT. Samples were then incubated at 37°C for 25mins. followed by cytocentrifugation onto glass slides. Two slides were prepared for each time point and 100 cells were examined to determine the proportion staining positively for NBT reduction.

5.1.6 *Treatment of cells with oligonucleotides.*

Nuclease-protected phosphorothioate oligonucleotides were synthesised at the Beatson Institute using standard phosphoramidite chemistry and were purified as described in the section below relating to purification of oligonucleotides. They were added daily to the tissue culture medium, for a period of 5 days at a final concentration of 20μM. The c-*myc* antisense oligonucleotide (AACGTTGAGGGGCAT) was targeted against the 5' of the mRNA and has been described previously (Wickstrom et al. 1988; Bacon and Wickstrom 1991). The other two oligos used in the study were: 1) the complementary c-*myc* sense oligonucleotide (ATGCCCCTCAACGTT) and 2) a random/degenerate oligonucleotide of the same length as the c-*myc* antisense oligonucleotide (ACTGACTGACTGACT).

## 5.2 Recombinant DNA techniques.

### 5.2.1 *Transformation of bacterial cells with plasmid DNA.*

DH5α competent cells were slowly thawed on ice and 50µl aliquots were placed into pre-chilled 2059 tubes (Falcon). Approximately 1ng of plasmid DNA was added to the cells and gently mixed by stirring with a pipette tip. After incubation of ice for 30min, the cells were heat-shocked at $42^0$C for 45sec and then placed on ice again for a further 2min. 450µl of L-broth was then added to the cells and this mixture was then incubated at 37°C for 1hr in an orbital shaker at 225rpm, to allow time for the cells to express the appropriate antibiotic resistance gene. After this time, 100µl of the cells was spread onto L-broth plates containing 1.5%(w/v) agar and supplemented with the appropriate antibiotic at a concentration recommended by Sambrook et al. (1989). Plates were then air-dried for 5 min and incubated in an inverted position for 16hr at $37^0$C.

### 5.2.2 *Small scale preparation of plasmid DNA.*

A single bacterial colony from an agar plate was picked using a sterile toothpick and transferred to approximately 5ml of L-broth medium containing the appropriate antibiotic. This medium was then incubated overnight at 37°C with shaking at 225rpm. 1.5ml of this culture was transferred to a microfuge tube and centrifuged at full speed for 30sec at 4°C. After removal of the supernatant, the bacterial pellet was resuspended in 100µl of solution A (50mM glucose, 25mM Tris.HCl (pH 8.0), 10mM EDTA). The cells were then lysed by addition of 200µl of freshly prepared solution B (0.2N NaOH, 1% SDS) and the contents mixed by gently inverting the tube 5 times. 150µl of solution C (2.5M KAc, 5M acetic acid, pH4.8) was then added to the tube, mixed by gentle vortexing for 10sec and placed on ice for 5min. The tube was then microfuged at 4°C for 5min at full speed and the supernatant transferred to a fresh tube. This supernatant was then extracted with an equal volume of TE-saturated phenol/chloroform as

recommended by Sambrook et al. (1989) and again placed into a fresh tube. The nucleic acids in this solution were precipitated by the addition of 2 volumes of ethanol followed by incubation at room temperature for 2min and tubes were then microfuged at 14,000rpm for 5 min. The resulting pellet was washed with 70% ethanol and air-dried for 10min. The nucleic acids were then dissolved in 50µl TE (10mM Tris.HCl (pH8.0), 1mM EDTA) containing DNAse-free RNAse at a final concentration of 20µg/ml. Typically 10µl of this mixture was then used for analysis in restriction digests.

5.2.3 *Large scale preparation of plasmid DNA.*

An overnight culture of bacteria, prepared in the same way as described above, was used to inoculate 500ml of L-broth medium containing the appropriate antibiotic. This culture was then once again incubated overnight at 37°C with shaking at 225rpm. The cells resulting from this incubation were then pelleted by centrifugation in a DuPont Sorvall RC3C centrifuge at 5,000rpm at 0°C for 10min. Pellets were thoroughly resuspended in 25ml solution A (see above) containing lysozyme at a final concentration of 5mg/ml and placed on ice for 30min. 40ml of freshly prepared solution B (see above) was then added to the cells, the solution mixed and placed again on ice for a further 5min. After this time, genomic DNA and proteins were precipitated by the addition of 20ml of solution C (see above), which was mixed into the lysed bacteria by inverting the tube gently 5 times. After being left on ice for 15min the flocculate resultant from the addition of solution C was pelleted by centrifugation in a DuPont Sorvall RC3C centrifuge at 5,000rpm for 5 min at 4°C. The supernatant was then filtered through a double layer of gauze, followed by precipitation of the nucleic acids by the addition of 0.6 volumes of propan-2-ol and incubation at room temperature for 15min. This solution was then centrifuged at 8,000rpm, 0°C in a Sorvall GSA rotor for 5 min. After being air-dried for 5min, the pellet was re-dissolved in 5.5ml TE containing

7.5g CsCl. Once dissolved 0.5ml of 10mg/ml ethidium bromide (Sigma) solution was added and the salt concentration of the mixture adjusted, by either addition of more water or more solid CsCl, to give a refractive index of 1.395. Samples were then transferred to screw-cap ultracentrifuge tubes and, making sure that opposing tubes were balanced to within 0.1g, centrifuged at 40,000rpm for >40hr. Plasmid bands were pipetted from the tubes and serially extracted with butan-1-ol to remove the ethidium bromide. They were then ethanol precipitated twice, washed with 70% ethanol, air dried and resuspended in approximately 1ml of TE (10mM Tris.HCl (pH8.0), 1mM EDTA).

5.2.4 *Storage of bacterial glycerol stocks.*

Stationary cultures in liquid medium were mixed with an equal volume of glycerol, chilled on ice and then stored at -20°C in plastic cryotubes. Cultures were re-established by inoculation of 5ml of L-broth medium, containing the appropriate antibiotic, with 20µl of the glycerol stock.

5.2.5 *Extraction and purification of mammalian genomic DNA.*

Approximately 5 x $10^7$ cells were washed twice in ice cold PBS and then lysed in 10ml of lysis buffer (10mM Tris.HCl (pH7.8), 0.5% SDS, 5mM EDTA) containing 50µg/ml proteinase K. Incubation was then undertaken with gentle mixing at 37°C for 16hr. The samples were then purified by extraction as follows: 2 times with 1 volume of TE-saturated phenol, once with 1 volume of TE-saturated phenol/chloroform and finally with 1 volume of chloroform/isoamyl alcohol (29 parts chloroform, 1 part isoamyl alcohol). The nucleic acids were then precipitated by adding 0.7 ml of 4M KAc and 30ml of ethanol, followed by incubation overnight at -20°C. The precipitated DNA was hooked from this solution and air dried before being placed at 4°C overnight in 2ml of resuspension buffer (50mM Tris.HCl (pH8.0), 10mM EDTA). DNAse-free RNAse was then

added to this solution at a final concentration of 0.1mg/ml and the sample incubated at 37°C for 2hrs. After this time, samples were extracted and precipitated as described above, air dried and finally resuspended in approximately 1ml of TE buffer. Samples were stored at 4°C until required.

5.2.6 *Synthesis and Purification of oligonucleotides.*

Oligonucleotides were synthesised at the Beatson Institute on an Applied Biosystems model 381A DNA synthesiser according to the manufacturers instructions. 5' trityl groups were removed as part of the synthesis and the oligonucleotides eluted into a solution of 29% (v/v) ammonia. This DNA-ammonia solution was then incubated at 55°C overnight in order to 'de-protect' the oligonucleotides. The vials containing this solution were then chilled on ice and the DNA-ammonia solution transferred to Falcon 2059 tubes. Oligonucleotides were precipitated by the addition of 0.1 volumes of 7.5M ammonium acetate and 3 volumes of ethanol, followed by incubation on dry ice for 30min. The DNA was then pelleted by centrifugation in a Sorvall HB-6 rotor at 10,000 rpm for 15min. The pellet was washed in 70% ethanol, air dried and dissolved in 0.5ml of de-ionised water. DNA concentrations were calculated as described below and the oligonucleotides were then stored at -20°C until required.

5.2.7 *Quantitation of nucleic acid concentrations.*

Nucleic acids were quantified with respect to their absorbance of UV light. 5µl of sample was added to 495µl of de-ionised water and the absorbance of the solution measured at 260nm and 280nm in a quartz cuvette, using de-ionised water as a blank. The concentration of the solution was calculated on the basis that an optical density of 1 corresponds to a concentration of 50µg /ml for plasmid or genomic DNA; 40µg/ml for RNA and 20µg/ml for single-stranded oligonucleotide. The ratio of the readings $A_{260}/A_{280}$ was used as an estimate of the purity of the

nucleic acid. Samples with ratios between 1.75 and 2.0 were taken as being sufficiently pure for all of the techniques undertaken in this study.

## 5.3 Restriction digests and electrophoresis.

### 5.3.1 *Restriction digests.*

Approximately 1μg of plasmid DNA for plasmid analysis or 15μg of mammalian genomic DNA for Southern analysis was digested in a final volume that was at least large enough to dilute the volume of enzyme added 10-fold. Plasmid digests were routinely carried out using 5 units of enzyme per μg of DNA in each reaction and were incubated at the recommended temperature for at least 1hr. Digests of genomic DNA were undertaken overnight with 10 units of enzyme being added for each μg of DNA to be digested. A fresh aliquot of enzyme (10 units/μg DNA) was added to genomic digests the following morning and the digest continued for a further 3 hrs, before the reaction was stopped by the addition of 1 volume of TE-saturated phenol/chloroform. Following this extraction, the genomic DNA was ethanol precipitated as described previously and then air dried prior to being resuspended overnight in an appropriate volume of TE for subsequent loading on an agarose gel. For digests of DNA involving two (or more) restriction enzymes, either a buffer compatible for both enzymes was used or the DNA was digested in sequential digests which were separated by phenol/chloroform extractions and ethanol precipitation.

### 5.3.2 *Agarose gel electrophoresis.*

DNA fragments from plasmids or genomic DNA were separated on non-denaturing agarose gels and visualised by staining with ethidium bromide, followed by UV transillumination. Typically, gels were prepared by dissolving 1% (w/v) electrophoresis grade agarose in 1x TBE buffer (90mM Tris.HCl, 90mM boric acid, 2mM EDTA, pH 8.0) or 1x TAE buffer (40mM Tris.acetate, 20mM

sodium acetate, 2mM EDTA, pH 7.4), containing 2.5 μg/ml ethidium bromide. After being heated in a microwave to dissolve the agarose, molten gels were cooled to approximately 60°C before being poured into an appropriate gel former. Once solid, gels were placed into electrophoresis tanks containing the appropriate 1x buffer and ethidium bromide as described above. Samples were mixed with one-sixth volume of gel-loading buffer (30% glycerol in water, 0.25% (w/v) bromophenol blue) prior to being loaded into the wells of the gel. Gels were initially subjected to 100V for 5-10min in order to run samples into the gel and the voltage then subsequently reduced to give a desired length of time for electrophoresis. As an estimation of the size of fragments resolved by electrophoresis, samples were run alongside molecular weight markers derived from the restriction digest of viral genomes. For large fragments, aliquots of a *Hind* III digest of bacteriophage λ were used and for smaller fragments aliquots of a *Hae* III digest of the bacteriophage ϕX174.

5.3.3 *Non-denaturing polyacrylamide gel electrophoresis.*

Non-denaturing polyacrylamide gels were used for the purification of DNA fragments and oligonucleotides and for the resolving of complexes in DNA-binding assays. A 30 % stock solution of acrylamide, comprising 29 parts acrylamide to 1 part N,N'methyl-bisacrylamide was diluted to give a 6% gel forming solution containing 0.5x TBE. This solution was polymerised by the addition of 250μl of 10% (w/v) ammonium persulphate and 100μl of TEMED per 50ml of gel solution. After mixing the solution was poured into vertical plates with 2mm spacing and allowed to set at room temperature. Gels were run at a constant current of 25mA and, where required, the DNA visualised by staining with a solution of 0.5μl/ml ethidium bromide for 10min, followed by washing in de-ionised water for 10min and UV transillumination. Gels used for DNA-binding assays were dried under vacuum at 80°C for 45min prior to autoradiography.

5.3.4 *Denaturing polyacrylamide gel electrophoresis.*

For the resolution of sequencing reactions, a gel solution was prepared which contained: 6% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 1x TBE, and 8M urea. Once the urea was completely dissolved the solution was polymerised by the addition of ammonium persulphate to a final concentration of 0.08% (w/v) and TEMED to a final concentration of 0.05% (v/v). This solution was then poured into plates (20cm x 45cm) which were separated by 0.5mm spacers and was allowed to set at room temperature. Gels were pre-run at ~1600V for 45min prior to the loading of samples and then the electrophoresis was continued for a further 2-3hr depending on the size of fragments to be resolved. The plates were then separated and the gel transferred to a sheet of Whatman 3MM paper, followed by drying under vacuum at 80°C for 2hrs prior to autoradiography.

5.3.5 *Sodium dodecyl sulphate polyacrylamide gel electrophoresis.*

The separation of proteins for immuno-blot analysis was undertaken using polyacrylamide gels containing SDS. The percentage of acrylamide used in the resolving gel depended on the molecular weight of the protein to be analysed, but was usually between 7-15%. The desired concentration of acrylamide was combined with 25ml of 4x gel buffer (1.5M Tris.HCl (pH 8.8), 0.4% (w/v) SDS) and water to a final volume of 100ml. The polymerisation of this solution was then initiated by the addition of 500µl of 10% (w/v) ammonium persulphate and 100µl of TEMED. To allow for the pouring of the stacking gel this solution was then cast to within ~3cm of the top of two glass plates which were separated by 2mm spacers. Prior to setting butan-1-ol was poured on top on the gel solution to eliminate any bubbles that would interfere with the formation of a smooth interface between the stacking and resolving gels. Once the gel was set, the butan-1-ol was washed away with de-ionised water. A stacking gel was then prepared which contained 4.8% (w/v) acrylamide, 0.125M Tris.HCl (pH 6.8), 0.1% SDS, 0.05% (w/v) ammonium

persulphate and 0.002% TEMED. This was poured on top of the resolving gel, a comb inserted and the solution allowed to set at room temperature for 15-20min. After polymerisation, the gel former, comb and gasket were removed and the glass plates containing the gel transferred to a gel tank containing 1x gel running buffer (50mM Tris.HCl, 1% (w/v) glycine, 0.25% (w/v) SDS). Unlike other acrylamide gels, SDS gels were not pre-run prior to the loading of samples as this disrupts the difference in salt concentrations between the resolving and stacking gels.

### 5.3.6 *Purification of DNA fragments from agarose gels.*

Bands were excised from agarose gels using a scalpel with the aid of UV transillumination. The agarose 'blocks' were then chopped into small pieces and the DNA extracted using a Geneclean kit as advised by the manufacturers. The purified DNA was then quantified by agarose gel electrophoresis alongside known quantities of viral DNA markers (e.g. $\phi$X174), which were used as stepwise gradients of DNA concentration.

### 5.3.7 *Purification of DNA fragments from polyacrylamide gels.*

Fragments were excised from polyacrylamide gels in the same way as described above. After excision, the gel slice was placed into a 0.5 ml eppendorf tube which had previously been pierced at its base with a small hypodermic needle. This tube was then placed into a 1.5ml tube and centrifuged at full speed for 5min in a microfuge. Two gel volumes of acrylamide elution buffer (0.5M ammonium acetate, 0.2% (w/v) SDS) was then mixed with the pulped gel slice and the tube incubated at 37°C for approximately 16hr. After this time, the tube was vortexed again and the contents loaded into a pierced 0.5ml eppendorf tube which had been plugged with a small amount of glass wool. This tube was placed as before in a 1.5ml eppendorf tube and microfuged at full speed for 5min. This effectively results in the retention of acrylamide in the glass wool, while DNA and elution buffer pass

through into the 1.5ml tube. The DNA sample was then further purified by a phenol/chloroform extraction and an ethanol precipitation, prior to being resuspended in an appropriate volume of TE buffer.

## 5.4 Polymerase chain reaction and DNA sequencing.

### 5.4.1 *cDNA synthesis.*

Approximately 5μg of total RNA in $H_2O$ was frozen on dry ice and lyophilised under vacuum. This was then re-dissolved in 18μl of annealing buffer (250mM KCl, 10mM Tris.HCl (pH8.3), 1mM EDTA), to which 1μg of reverse transcription primer (minus strand primer) was then added. This RNA-oligonucleotide mixture was then placed at 83°C for 3min in order to denature the RNA template and then incubated at 70°C for 25min to allow for annealing of the oligonucleotide to its complementary target sequence(s). After this time the tube was transferred to a 1000ml glass beaker containing water at 70°C which was then placed at room temperature and allowed to cool slowly to 45°C. The tube was then briefly microfuged and the contents combined with 30μl of cDNA buffer (24mM Tris.HCl, 16mM $MgCl_2$, 8mM DTT, 0.4mM of each of the four dNTPs (dATP, dCTP, dGTP, dTTP)) and 1μl (20 units) of AMV reverse transcriptase. After incubation at 43°C for 1hr, the cDNA was precipitated by the addition of 0.5 volumes of 7.5M ammonium acetate and 2 volumes of ethanol, followed by incubation on dry ice for 1hr or at -20°C overnight. Nucleic acids were then pelleted by centrifugation at full speed in a bench top microfuge for 15min at 4°C and were subsequently air dried and re-suspended in 20μl of $dH_2O$. This reaction typically gave enough cDNA for 2 PCR reactions. For each cDNA synthesis reaction a corresponding reaction was also undertaken which did not contain reverse transcriptase. This reaction was then later used as a control in PCR reactions to show that any products which were amplified in the reverse

transcriptase positive reaction had arisen from an RNA template and not from contaminating DNA in the original RNA sample.

5.4.2 *Polymerase chain reactions.*

Polymerase chain reactions were undertaken using a Geneamp kit from Perkin Elmer Cetus. Half of the products (10µl) from a cDNA synthesis reaction were added to a 100µl PCR reaction which contained 1x PCR buffer (10x buffer is 500mM KCl, 100mM Tris.HCl (pH8.3)), 2mM $MgCl_2$, 0.2µM forward primer, 0.2µM reverse primer and 2.5units of *Taq* polymerase. The components of this reaction were mixed and over-layed with ~60µl of paraffin oil to stop evaporation of the contents of the tube during amplification. Amplifications were undertaken on a DNA thermal cycler (Perkin Elmer Cetus) for 35 cycles (1 cycle was typically: denaturation at 94°C for 1min, annealing at 55°C for 1 min, extension 72°C for 2min.), followed by incubation at 72°C for 15min in order to ensure that all products were completely extended. Reactions were extracted with 1 volume of chloroform and the products then analysed by agarose gel electrophoresis. If, in addition to the desired product, many other DNAs were also amplified, the specificity of the reaction was increased in one of two ways. Either the annealing temperature was increased to 57°C or 60°C, or the concentration of $MgCl_2$ was reduced in a stepwise manner to as low as 1.25mM. If neither of these two actions increased the specificity of the reaction a different set of amplification primers were designed.

5.4.3 *Direct sequencing of PCR products.*

For the direct sequencing of PCR products, amplifications were undertaken using primers which had been modified to include a 5' biotin group. Biotinylated reverse primers and standard forward primers were used for amplification of products for which sequence was required in the sense orientation.

The reverse combination was used when sequencing was required in the antisense direction. Separation of the two strands from each PCR reaction was undertaken by binding the product to streptavidin labelled iron beads (Dynal) as recommended by the manufacturer, followed by melting of the DNA duplex in 0.1M NaOH and separation of the two strands by magnetic attraction of the iron beads. The immobilised strand was then annealed to a suitable sequencing primer in a reaction containing ~1μg of immobilised product, 20ng primer, 0.5% NP40 and 1x annealing buffer (5x annealing buffer is: 200mM Tris.HCl (pH7.5), 100mM $MgCl_2$, 250mM NaCl) made up to a final volume of 14μl with $dH_2O$. This mixture was boiled for 5min and then snap frozen in a dry-ice/ethanol bath for 2min. Products were then sequenced using a Sequenase kit according to the manufacturer's instructions. Immediately prior to being resolved by electrophoresis on urea-containing polyacrylamide gels, samples were denatured by heating to 95°C for 5min.

5.4.4 *Automated chain terminator sequencing.*

Both cloned DNA and PCR products were sequenced using an Applied Biosystems ABI 373A automated DNA sequencer. 0.3-0.5μg of plasmid DNA or 50-100ng of a PCR reaction was mixed with 20ng of sequencing primer and 8μl of 'Dyedeoxy' reaction mix in a total reaction volume made up to 20μl with $RQH_20$. DNA was amplified in a DNA thermal cycler (Perkin Elmer Cetus) for 25 cycles (1 cycle was: 15sec at 96°C to denature DNA, 1sec at 50°C for annealing and 4min at 60°C to extend), and the products were then precipitated by the addition of 0.1 volumes of 3M sodium acetate (pH 4.5) and 2.5 volumes of ethanol. Pellets were washed with 70% ethanol and air dried prior to being re-suspended in loading buffer and subjected to electrophoresis as advised by the manufacturers.

## 5.5 Isotopic labelling of DNA probes.

### 5.5.1 *Preparation of random-primed radio-labelled probes.*

The probes used in this study for the analysis of Northern and Southern blots were obtained as follows:

The c-*myc* probe was the 1.4 kb Cla1-EcoR1 fragment of the plasmid pMC41-3RC (Watson et al. 1983) comprising the third exon and 3' flanking sequence of the human c-*myc* gene.

The *max* probe was a 500bp fragment of the plasmid pSTMax7 (a kind gift of Drs. D.H. Crouch and D.A.F. Gillespie of the Beatson Institute) containing the entire coding region of the human *max* gene and has been described previously (Crouch et al. 1993).

The *mad* probe was a 1kb fragment of the plasmid pVZMad (a kind gift provided by Dr. D. Ayer, Fred Hutchinson Cancer Research Center, Seattle, USA) containing the entire coding region of the human *mad* mRNA.

The *ODC* probe, which was gift of Tam Jamieson of the Beatson Institute, was a full length cDNA clone of the human ornithine decarboxylase gene and has been described previously by Hickok et al. (1990).

The *MRP14* probe used was a partial cDNA clone that was previously isolated in our group by Sheila Graham from a chronic granulocytic leukaemia cDNA library which had been constructed by Drs L. Wiedermann and R. Tindle, and has been described previously as 2B5 (Graham and Birnie 1988).

The probe used to analyse the expression of c-*myb* was kindly provided by Pierre Schembri-Wismayer of the Beatson Institute and has been described previously as pMbm I by Clarke et al. (1988).

The mouse *GAPDH* clone was kindly provided by Max Walker of the Beatson Institute and comprises 270bp of the coding region of the mRNA.

The $\beta_2$-microglobulin probe comprises 97% of the coding region plus 3' untranslated sequence of the human cDNA which was previously cloned into the Pst1 site of pBR322 (Suggs et al. 1981).

The 28S ribosomal RNA probe was a 7.4kb EcoR1 fragment of the plasmid pHR-28-1 and has been described previously by Erickson et al. (1981).

The *mxi1* probe was generated from uninduced HL60 RNA via RT-PCR using the primers: 5'-GGAGCGAAGAGTGTGAACAT-3' (forward); 5'-TACTCAATGTAGTATGAG-3', which results in the amplification of the entire coding region of the mRNA.

All probes were radio-labelled with $^{32}$P-dCTP via random priming using a kit supplied by Boehringer Mannheim. Following each labelling reaction, probes were separated from unincorperated nucleotides by purification on Nick columns as described in the manufacturers instructions. Before being used in hybridisation reactions, all double-stranded probes were denatured by heating to 100°C for 5 min and then placed on ice for a further 10min.

5.5.2 *Radio-labelling of oligonucleotides.*

Complimentary single-stranded oligonucleotides were combined at equimolar ratios in TE buffer and heated to 95°C for 5min in a large volume of $H_20$. After this time the water bath was allowed to slowly cool to room temperature in order for effective annealing of the two oligonucleotides to occur. The resulting double-stranded oligonucleotides were then end-labelled using bacteriophage T4 polynucleotide kinase (PNK). 300ng of oligonucleotide per 50µl reaction was incubated in 1x T4 PNK buffer (10x PNK buffer is: 0.5M Tris.HCl (pH 7.6), 0.1M $MgCl_2$, 50mM dithiothreitol (DTT), 1mM spermidine, 1mM EDTA), containing 50µCi [$\gamma$-$^{32}$P] ATP and 20 units of PNK enzyme. Reactions were carried out at 37°C for 1hr and were then stopped by the addition of 2.5µl of 0.25M EDTA. Probes were subsequently separated from the unincorporated label by polyacrylamide gel electrophoresis and then purified as described in the section relating to purification of DNA fragments from polyacrylamide gels.

**5.6 Northern and Southern blotting.**

5.6.1 *Northern blot analysis.*

Total cellular RNA was isolated using RNAzol B, as advised by the manufacturers. RNA was denatured by suspension in 50% formamide, 2.2 M formaldehyde in 40mM 3-(N-morpholino)propane sulphonic acid, 10mM sodium acetate, 1mM EDTA, pH 7.0 and heating at 65°C for 15 min. 15µg of RNA per lane was size-fractionated by electrophoresis through 1.25% agarose gels containing 2.2 M formaldehyde. Gels were washed in water (2 x 30min) followed by transfer to Hybond N+ membranes via capillary blotting using 20 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7). Filters were then rinsed in 2x SSC and the RNA fixed to the membrane by exposure to UV light in a Stratagene 1800 crosslinker. While still damp filters were sealed in polythene bags and stored at 4°C until required

5.6.2 *Southern blot analysis.*

DNA which had been digested with the appropriate restriction enzyme was loaded onto a 1% agarose gel containing 1x TBE, 0.5μg/ml ethidium bromide. Samples were then subjected to electrophoresis for ~16hr at 30V. Once photographed using UV transillumination, the gel was soaked in 1.5M NaCl, 0.5M NaOH for 2x 20min with gentle shaking and then for a further 2x 30min in 1M ammonium acetate, 0.02M NaOH. This buffer was then used to transfer the DNA to Hybond N+ nylon membrane overnight via standard capillary blotting as described by Sambrook et al. (1989). Filters were then washed and crosslinked as described previously for RNA blots.

5.6.3 *Hybridisation.*

Nylon membranes were pre-hybridised in a solution of 50% formamide, 5 x SSPE (1 x SSPE is 0.18 M NaCl, 0.01 M sodium phosphate pH7.4, 0.1mM $Na_2EDTA$), 5 x Denhardt's reagent (see Sambrook et al. 1989), 0.5 % (w/v) SDS and 200μg/ml sonicated salmon sperm DNA at 42°C for 4 hr. Radio-labelled probe was then added to this solution at a concentration of 1 x $10^6$ cpm/ml and the incubation continued for a further 16 to 24hr. The blots were then washed (2 x 20 min in 2 x SSC, 0.1% SDS at room temperature; then 45min in 0.1 x SSC, 0.1% SDS at 65°C), and exposed to Kodak X-OMAT film with intensifying screens at -70°C. Autoradiographs were quantified by scanning laser densitometry using apparatus from Molecular Dynamics and software from PDI.

5.6.4 *Removal of radio-labelled probe from nylon membranes.*

In order that nylon membranes could be re-hybridised with a series of different probes, bound probe was removed by washing in solutions of boiling SDS. 0.5% (w/v) SDS for Southerns and 0.1% (w/v) SDS for Northerns was boiled and poured onto membranes which were then shaken at room temperature until the SDS

solution had cooled to room temperature. Blots were then re-sealed into polythene bags and stored at 4°C until required. At no point during this procedure were the blots allowed to go dry as this causes the probe to become irreversibly bound to the nylon membranes.

## 5.7 Protein analyses and flow cytometry.

### 5.7.1 *Western blot analysis.*

Samples of cells from the various time points indicated were harvested by centrifugation or scraped from the tissue culture dish and subsequently washed twice in ice-cold PBS. They were then lysed in SDS-PAGE sample buffer (1x sample buffer is 20% (v/v) glycerol, 2% (w/v) SDS, 100mM Tris.HCl pH6.8 and added immediately prior to lysis 5% β-mercaptoethanol) and boiled for 5mins prior to being chilled on ice. Lysates were then sonicated in order to shear high molecular weight DNA and then centrifuged at 14,000 rev/min for 2min at 4°C in a microfuge. The resultant supernatants were stored in aliquots at -70°C until required.

Equal quantities of protein from each time point were separated by electrophoresis at 30mA for 16hr through 7% SDS-polyacrylamide gels (30% acrylamide stock solution: 29.6% [w/v] acrylamide, 0.4% [w/v] bisacrylamide). Pre-stained 'rainbow' markers were run alongside samples as a means of assessing the extent of electrophoresis and for the subsequent calculation of the molecular weight of proteins detected in the Western blotting. The proteins from these gels were transferred to Transblot nitro-cellulose membrane via semi-dry electro-blotting at 180mA for 1hr using layers of Whatman 3MM paper which had been soaked in semi-dry blot buffer (60mM Tris.HCl, 50mM glycine, 1.6mM SDS, 20% (v/v) methanol. Before the membranes were taken through the immuno-blot protocol, the efficiency of transfer and the relative loading per lane was assessed by staining the membrane with Ponceau S stain. 10ml of 1x stain (10x stain is: 2% (w/v) Ponceau

S, 30% (w/v) trichloroacetic acid, 30%(w/v) sulphosalicyclic acid in $dH_20$), was placed on the membrane for ~3-5min and was then rinsed away with $dH_20$ until the protein bands became visible. Once analysed, the remainder of the stain was washed away with copious amounts of $dH_20$ before the membrane was probed as outlined below.

Detection of c-Myc protein on these blots was undertaken using an antibody raised against human c-Myc, which was a kind gift of Dr. D. Gillespie of the Beatson Institute and which has been described previously (Crouch et al. 1993; La Rocca et al. 1994). Blots were first blocked for 16hr in PBS containing 5% (w/v) non-fat milk powder, 0.05% Tween 20, and then subsequently incubated in this solution with the c-Myc antiserum for 1hr, then with an a anti-rabbit horseradish peroxidase conjugated secondary antibody for 1hr. The extent of reactivity against c-Myc was then ascertained by using the ECL Western blotting system and exposure to Kodak X-OMAT film.

5.7.2 *Electrophoretic mobility shift assays.*

Typically 1 x $10^7$ cells were used for the isolation of nuclear proteins for each sample and were prepared and stored as of the protocol by Andrews and Faller (1991). To analyse the DNA binding activities during the differentiation programmes, 10μg of nuclear protein from each time point was incubated with 0.2 ng of $^{32}P$ end-labelled 'CM1' double-stranded oligonucleotide (5'-CCCCCACCACGTGGTGCCTGA-3', which has been selected as a consensus Myc:Max binding site (Blackwood and Eisenman 1991), at room temperature for 20 min, in a buffer consisting of 20mM HEPES (pH 7.2), 50 mM KCl, 1mM EDTA, 3mM $MgCl_2$ and 10% glycerol. 200ng of an unrelated double-stranded oligonucleotide (5'-TTCCGGCTGACTCATCAAGCG-3') was used as a competitor in each reaction to sequester non-specific DNA binding proteins. Where indicated, the binding reactions were also incubated with either competitor DNA or

antibody for 30 min on ice prior to the addition of the labelled probe. Subsequently, binding reactions were resolved by electrophoresis through a 6% (0.5x TBE) polyacrylamide gel for approximately 3hr at 25mA. The gel was then dried and exposed to Kodak X-OMAT film with intensifying screens at -70°C. Antibodies used in these reactions were obtained as follows. MadAb and MaxAb1 were from Santa Cruz Biotechnology, Inc. MaxAb2 and Myc sera were a kind gift from David Gillespie of The Beatson Institute, Glasgow and have been described previously (Crouch et al. 1993; La Rocca et al. 1994). MaxAb3 was a kind gift from Trevor Littlewood and Gerard Evan of the Imperial Cancer Research Fund, London and has been described previously by Littlewood et al.(1992) as MX.

5.7.3 *Flow cytometry.*

At the indicated times post-treatment, both adherent and suspension cells were harvested and washed twice in ice-cold PBS. The cells were then fixed by resuspension in 70% ethanol to give a final concentration of approximately $10^6$/ml and stored at 4°C until required. To assess the cell-cycle status of each population of cells, stored samples were centrifuged and resuspended once again to a concentration of $10^6$/ml in 15mM $MgCl_2$, 0.002% (w/v) Chromomycin A3. After incubation in this stain at room temperature for 30min in the dark, these samples were analysed using a Fluorescence-Activated Cell Sorter (FACStar, Becton Dickinson). Equal numbers of events were analysed for each population of cells and cell-cycle related DNA profiles and their analyses were undertaken using the Consort system (Becton Dickinson).

# RESULTS

## CHAPTER 6: Analysis of the changes in abundance of the mRNAs for c-*myc*, *max*, *mad* and *mxi1* during processes of myeloid differentiation.

### 6.1 *Analysis of differentiation in the HL60 cell line.*

As described previously in Chapter 3, the HL60 leukaemic cell line can be maintained in culture as a population of cells arrested at the promyelocytic stage of differentiation (Birnie 1988). Release of this differentiation block, resulting in differentiation to either monocytes/macrophages or granulocytes has been shown to occur as the result of treatment with a variety of physiological and chemical inducers (Collins 1987). During the course of the study, differentiation to monocytes/macrophages was achieved by treatment with either TPA for 3 days at a final concentration of $1.6 \times 10^{-7}$M or with 1-α,25-dihydroxyvitamin D3 (Vit D3) for 5 days at a final concentration of $1 \times 10^{-7}$M. As a quantitative assessment of the effect of treating the cells with these inducers the cells were stained for the activity of the terminal differentiation marker, non-specific esterase (NSE). As can be seen in Figure 9, treating the cells with TPA resulted in a rapid increase of the number of cells exhibiting an activity for this marker, with in excess of 80% staining positive after only 24hrs of exposure to TPA. This increased by approximately 5% over the next 24hr period and was then maintained at this level until the cells ultimately died 72hrs after the addition of the TPA. In contrast, when the cells were differentiated with Vit D3, the number of cells staining positive for NSE increased much more slowly (Figure 9). In fact, 5 days of treatment was required to cause the same number of cells to show activity for the marker (~80%) as was seen following treatment with TPA for a period of only 24hr.

During this study, differentiation of HL60 cells was also induced to granulocytes by treatment with DMSO for a period of 5 days. As a measurement of the number of cells which had terminally differentiated, the cells were assayed for

Figure 9

Figure 9: *Percentage of cells staining positively for markers of terminal differentiation during HL60 cell differentiation.*

HL60 cells were analysed for their ability to reduce nitroblue tetrazolium (NBT) during granulocytic differentiation induced by DMSO and for the activity of non-specific esterase (NSE) during monocytic differentiation induced by TPA and 1-α, 25-dihydroxyvitamin D3. Analysis of differentiation induced by TPA was only taken up to 3 days, as the cells die beyond this time point. These findings were reproducibly seen in at least two individual experiments for each individual time point.

Percentage of cells staining positive for markers of terminal differentiation
90
80
70
60
50
40
30
20
10
0
0hr
12hr
24hr
36hr
48hr
60hr
72hr
84hr
96hr
108hr
120hr
Time following treatment with the differentiation inducer (Hours)
TPA
DMSO
Vit D3

their capacity to produce superoxide. This assay, which has been described previously (Freeman and King 1972), is based on the ability of the cells to reduce nitroblue tetrazolium which results in the formation of visible NBT-formazan deposits in the cytoplasm of the cells. Analysis of this marker during the differentiation programme revealed that the accumulation of terminally differentiated cells was a gradual process over the 5 day incubation period (Figure 9). However, after this time, it was routinely found that more than 80% of the cells scored positive in this assay.

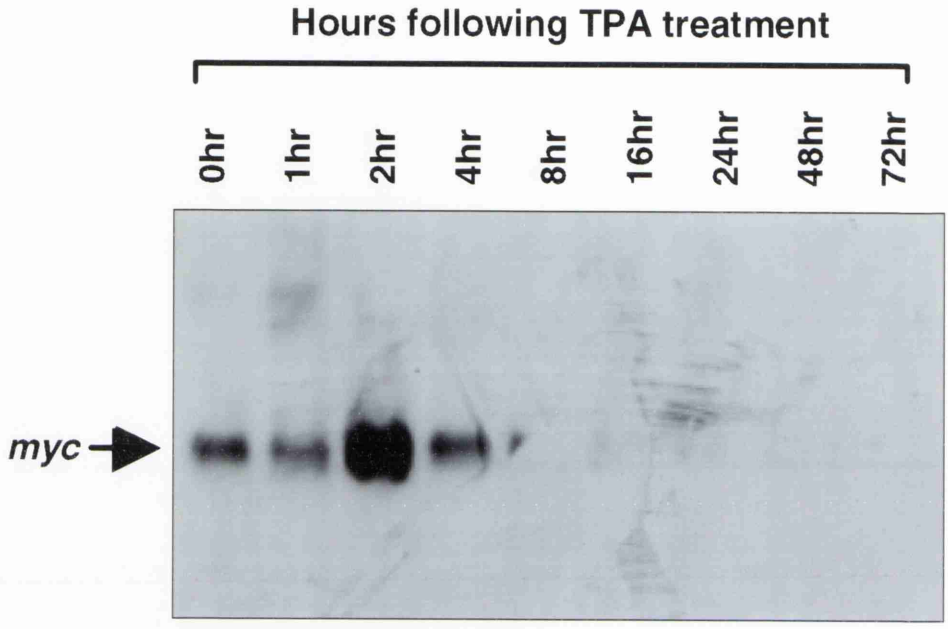
## 6.2 *Analysis of the changes in the levels of c-Myc protein during HL60 differentiation.*

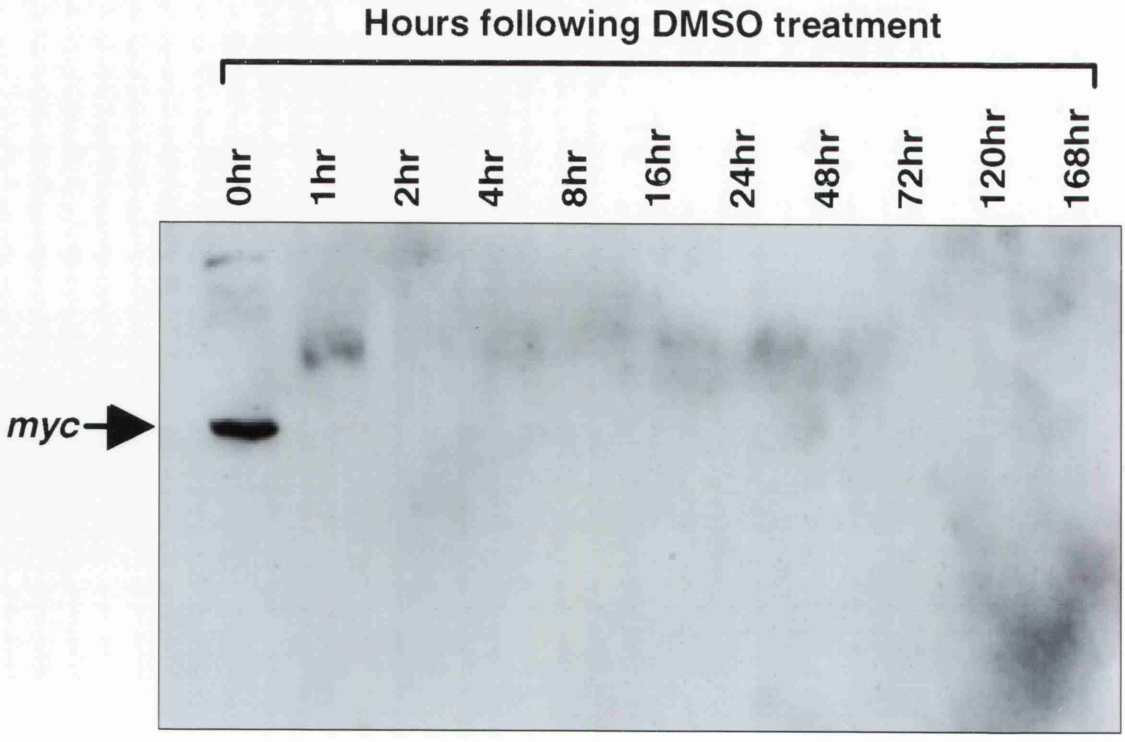
Both ourselves (Mitchell et al. 1992) and others (Westin et al. 1982; Reitsma et al. 1983) have shown previously that down-regulation of the levels of c-*myc* mRNA is a rapid event during induced differentiation of HL60 cells. To assess whether these observed changes in mRNA abundance were reflected in changes in the levels of the c-Myc protein, Western blot analysis was undertaken on protein samples taken at various points during the differentiation programmes. As can be seen in Figure 10, loss of c-Myc protein was indeed an early event following treatment with the differentiation inducers. Moreover, the time frame over which the protein was lost was found to be almost identical to that which was observed for c-*myc* mRNA (Mitchell et al. 1992 & Figure 11). During differentiation to monocytes/macrophages, the levels of the protein initially underwent a transient increase of approximately 5-fold (as determined by densitometric analysis) after 2hrs of treatment with the differentiation inducer. The abundance of the protein was then down-regulated to undetectable levels between 4 and 8 hrs after addition of the phorbol ester and were then maintained at these levels for the remainder of the differentiation programme. As indicated above, changes in the levels of the mRNA

Figure 10

Figure 10: *Western blot analysis of the levels of c-Myc protein during differentiation of HL60 cells induced by DMSO and TPA.*

Western blot analysis of the levels of c-Myc protein in HL60 cells treated for the times indicated with TPA at a final concentration of $1 \times 10^{-7}$ M or DMSO at a final concentration of 1.5% (v/v). Equal quantities of protein from each time point were resolved through polyacrylamide gels, blotted and assessed for Myc protein using an antiserum which had been raised against the full-length human c-Myc protein.


Hours following TPA treatment
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
myc


Hours following DMSO treatment
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
120hr
168hr
myc

were very similar, with a transient 2-3 fold increase (as determined by densitometric analysis) occurring after two hours of treatment, followed by a rapid down-regulation of the message between the 2 and 4 hr time points.

The analysis of the changes in the abundance of the c-Myc protein during DMSO-induced granulocytic differentiation revealed a pattern of change that was again very similar to the changes in the levels of c-*myc* mRNA. In contrast to the changes observed during differentiation to monocytes/macrophages, no transient increase in either the message (Mitchell et al. 1992 & Figure 11) or the protein (Figure 10) were observed following addition of the differentiation inducer. Both the abundance of the message and the protein were rapidly down-regulated during the first hour of the differentiation programme and then remained at this level or lower for the next 7 days.

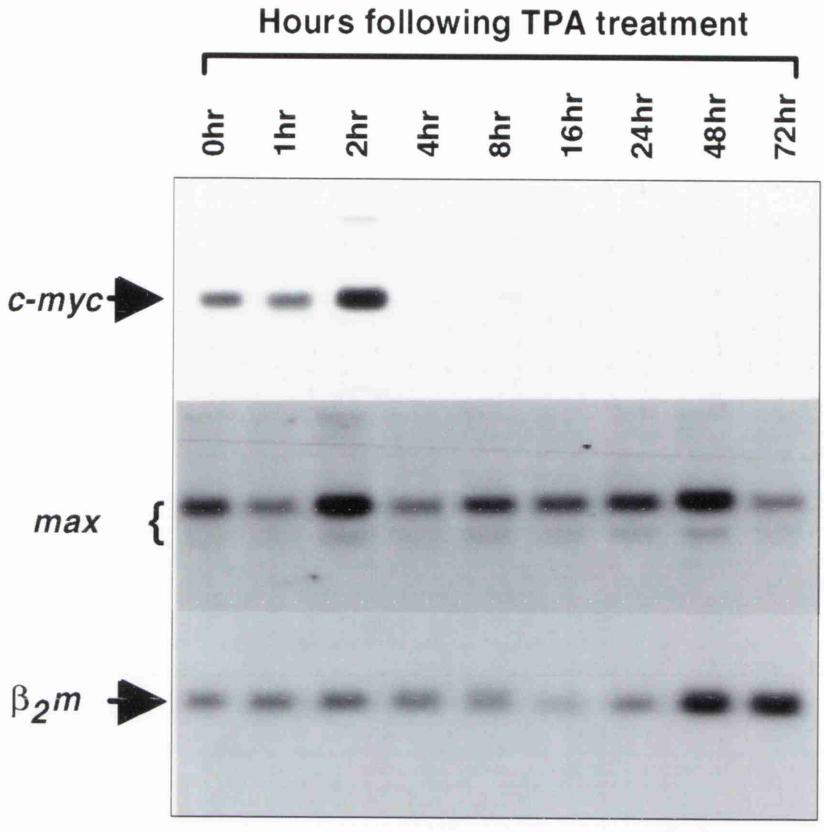
### 6.3 *Analysis of the changes in abundance of max mRNA during differentiation in HL60 cells.*

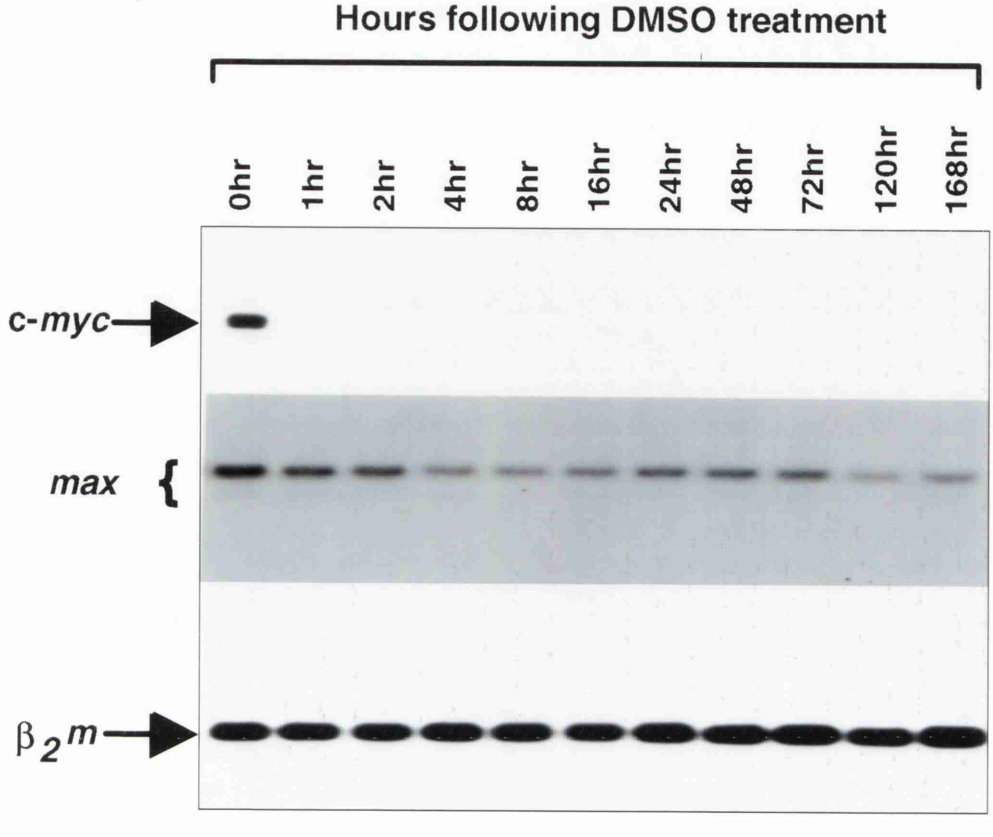
Prior to the start of this study, it had been shown that effective DNA-binding by c-Myc was dependent on it forming a heterodimeric complex with a second protein, Max (Blackwood and Eisenman 1991). In addition, it is considered that c-Myc brings about its phenotypic effects through transactivation of its target genes (Luscher and Eisenman 1991). Therefore, it was not unreasonable to assume that any changes in the abundance of Max protein would affect the correct functioning of c-Myc. We therefore examined the changes in the levels of *max* mRNA, relative to those of c-*myc*, during the induced differentiation of HL60 cells. Northern blots of RNA isolated from HL60 cells at various times during the differentiation programmes were hybridised with a full-length cDNA probe of the coding region of the human *max* mRNA (a gift from Drs. D.H. Crouch and D.A.F. Gillespie of the Beatson Institute). This probe was found to hybridise to two RNA

Figure 11

Figure 11: *Northern analysis of the levels of c-myc and max mRNAs during differentiation of HL60 cells induced by DMSO and TPA.*

Northern blot analysis of RNAs isolated from HL60 cells which had been treated with either TPA (A) at a final concentration of $1.6 \times 10^{-7}$M or DMSO at a final concentration of 1.5% (v/v). RNA was isolated at the times indicated following addition of the differentiation inducer. The blots were sequentially hybridised with probes for c-*myc*, *max* and $\beta_2$–*microglobulin* and sections are labelled accordingly. Effective detection of the smaller *max*-hybridising RNA species routinely required much longer periods of autoradiography than those used to create the figure shown.


Hours following TPA treatment
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
c-myc
max
β2m


Hours following DMSO treatment
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
120hr
168hr
c-myc
max
β2m

species as has been described previously (Blackwood at el. 1992). The larger and more abundant of these two species had an apparent size of 2kb, as compared to 1.8kb for the smaller species which routinely required much longer periods of autoradiography for effective detection. For comparison, these blots were then subsequently stripped and sequentially re-hybridised with probes for c-*myc* and $\beta_2$-*microglobulin* (see Methods 5.5.1).

When analysis was made of the levels of *max* mRNA during differentiation to granulocytes following treatment with DMSO (Figure 11), it was apparent that the message was regulated in a very different manner from that of c-*myc*. While, as described previously, c-*myc* was down-regulated very rapidly following addition of the differentiation inducer (Mitchell et al. 1992), the levels of *max* mRNA fluctuated only slightly and remained evident for the whole of the differentiation time course. In contrast, when comparison was made between the patterns of c-*myc* and *max* mRNA abundance during TPA-induced differentiation to monocytes/macrophages a different pattern of changes in the levels *max* was observed. After an initial slight decrease in abundance of *max* mRNA following 1hr of TPA treatment, the levels were then increased to approximately 2 fold higher (as assessed by densitometric analysis) than that observed in untreated cells (Figure 11). Although this increase was very similar to that observed for c-*myc* mRNA, *max* mRNA was not subsequently rapidly down-regulated after this 2hr time point. In fact, the levels continued to undergo some slight fluctuations and were only decreased at the late stages (48hr-72hr after TPA treatment) of the differentiation programme. However, when this decrease was normalised against the levels of $\beta_2$-*microglobulin* mRNA, it was found that the abundance of *max* mRNA at the 72hr time point was 9-fold lower than that observed in untreated cells (values were determined by laser densitometry).

6.4 *Comparison of the stability of max mRNA in untreated and TPA-treated HL60 cells.*

To investigate whether the down-regulation of *max* mRNA in TPA-treated HL60 cells could be explained, at least in part, by a decrease in mRNA stability, analysis was made of the stability of *max* in both untreated cells and cells treated with TPA for 72hr. Cultures of HL60 cells were incubated in the presence of either 1.6 x $10^{-7}$M TPA or a corresponding amount of solvent for 72hr. Actinomycin D (Act D) was then added to the cultures at a final concentration of 10 μg/ml in order to prevent new mRNA synthesis (Sambrook et al. 1989). Blots of RNA isolated at the times indicated following Act D treatment were first hybridised with the *max* cDNA clone described above and were then stripped and re-probed with a probe for *28S* RNA as assessment of the amount of RNA loaded in each lane. *28S* RNA was used instead of $\beta_2$-*microglobulin* as ribosomal RNAs are unaffected by treatment with actinomycin D (Alberts et al. 1989).

Initial analysis of these Northern blots indicated that the stability of *max* mRNA appeared to be unaffected by treating the cells with TPA (Figure 12). To confirm that this was the case the blots were scanned using a laser densitometer and the values obtained for the abundance of *max* mRNA at each time point normalised against the values obtained for *28S* RNA. These values were then plotted against time as is shown in Figure 13. Interpolation of these graphs showed that the half-life of *max* mRNA was 2.3hrs in untreated cells and 2.1hrs in cells treated with TPA for a period of 3 days. These findings therefore show that the stability of *max* mRNA is moderately reduced by treating the cells with TPA. However, although this decrease in stability may well be a contributing factor in the down-regulation of the levels of *max* mRNA, as the difference is so small, it seems unlikely that it is the sole factor involved in this process. As a result it is tempting to speculate that the levels of the mRNA are to the greater extent transcriptionally

Figure 12

Figure 12: *Analysis of the effects of TPA treatment on the stability of max mRNA of HL60 cells.*

Northern blot analysis of RNAs isolated from HL60 cells which had been maintained for 72hr either in the absence (A) or presence (B) of TPA at a final concentration of $1.6 \times 10^{-7}$M and then subsequently treated with actinomycin D to a final concentration of 10μg/ml for the times indicated. The blots were sequentially hybridised with probes for *max* and $\beta_2$–*microglobulin* and sections are labelled accordingly.

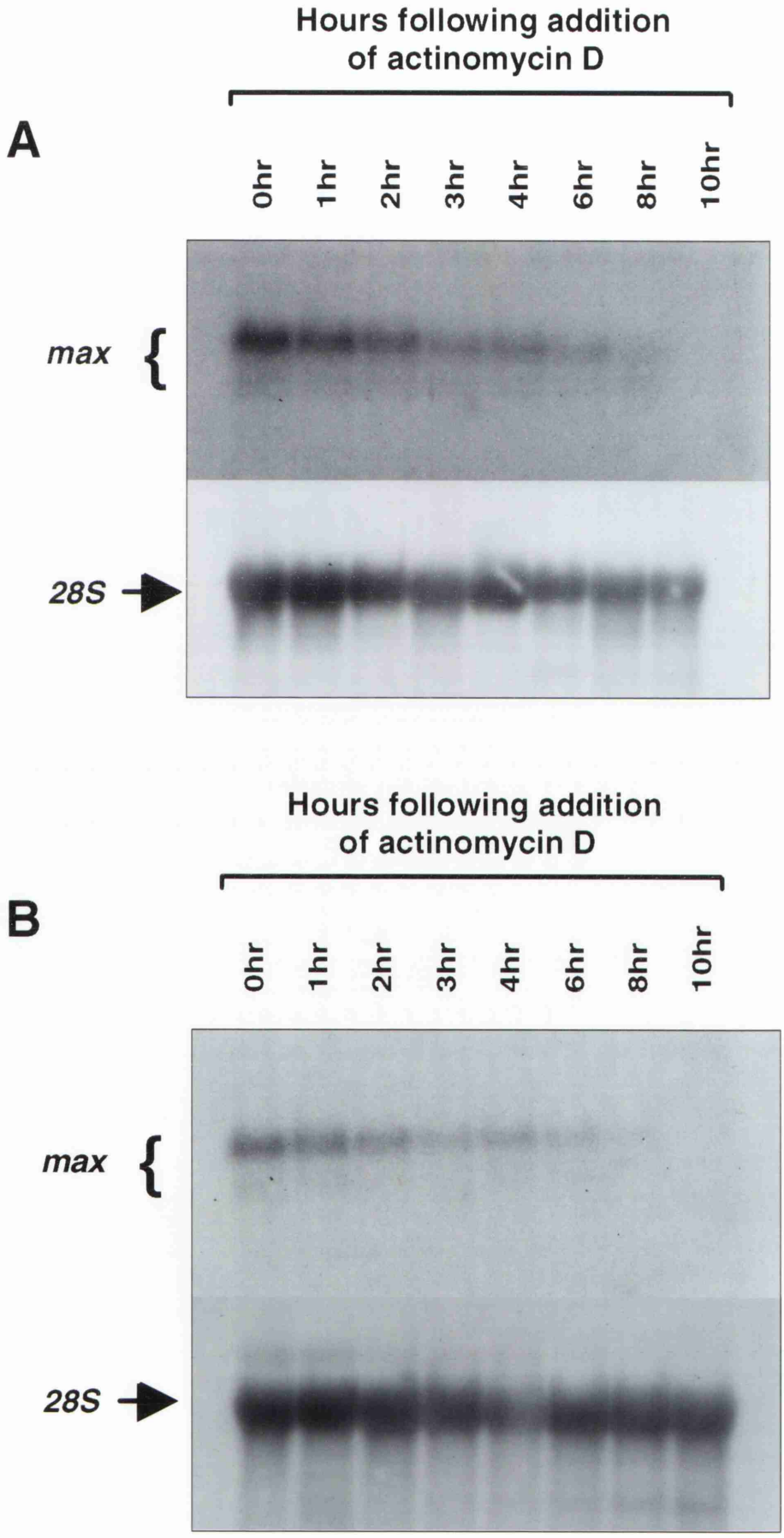
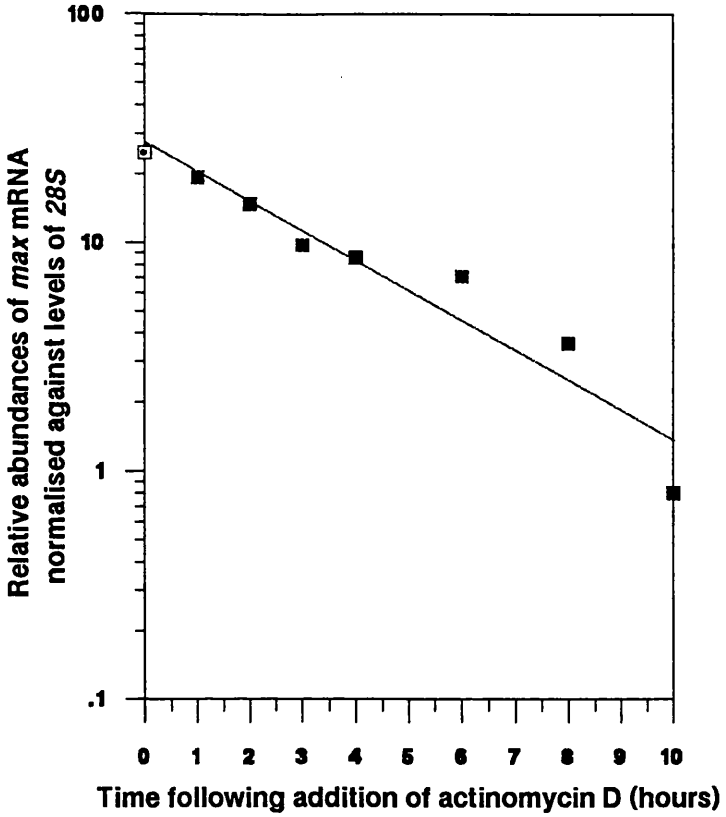

A
Hours following addition of actinomycin D
0hr
1hr
2hr
3hr
4hr
6hr
8hr
10hr
max
28S
B
Hours following addition of actinomycin D
0hr
1hr
2hr
3hr
4hr
6hr
8hr
10hr
max
28S

Figure 13

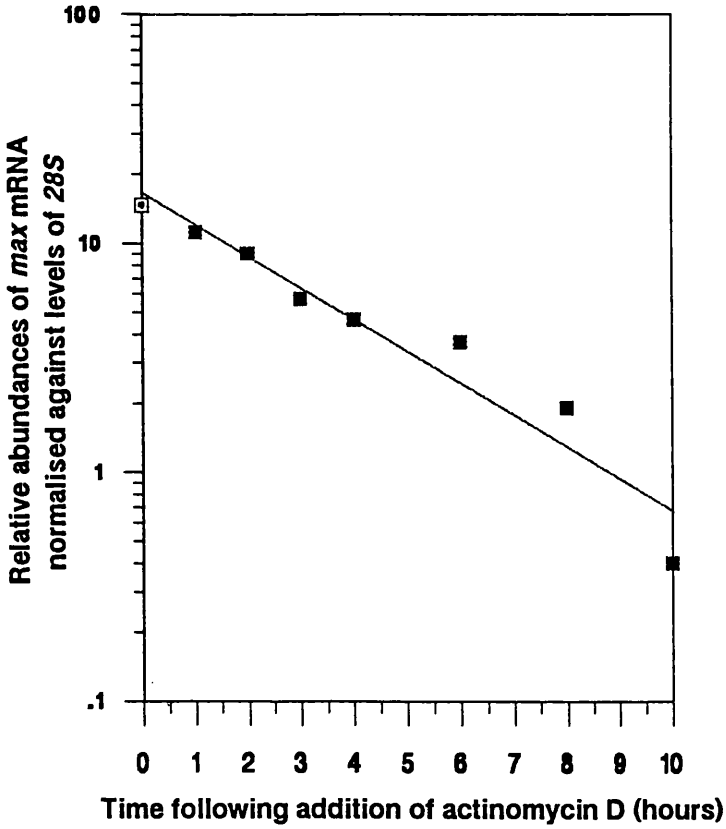
Figure 13: *Scanning laser densitometric analysis of the Northern blots in Figure 4.*

The blots in figure 4 were scanned using a scanning laser densitometor and the values obtained for the levels of *max* mRNA normalised against the relative levels of *28S* RNA in each individual sample. Panel A represents values obtained from untreated HL60 cells. Panel B represents the values obtained from HL60 cells treated for 72hrs with TPA prior to being treated with actinomycin D. The values for the relative abundance of *max* at each time point is measured in arbitrary units.

A


100
10
1
.1
Relative abundances of max mRNA normalised against levels of 28S
0 1 2 3 4 5 6 7 8 9 10
Time following addition of actinomycin D (hours)

B


100
10
1
.1
Relative abundances of max mRNA normalised against levels of 28S
0 1 2 3 4 5 6 7 8 9 10
Time following addition of actinomycin D (hours)

down-regulated. However, nuclear 'run-on' transcription assays would be required to confirm that this was the case.

### 6.5 *Analysis of max mRNA in murine bone marrow-derived macrophages.*

It has been shown that stimulation of murine bone marrow-derived macrophages (BMM) with M-CSF results in cell-cycle progression and a concomitant increase in the levels of c-*myc* mRNA (Hamilton et al. 1989). As we found that the abundance of *max* mRNA is markedly decreased during monocytes/macrophage differentiation in HL60 cells (Figure 11), we wondered if the levels of *max* mRNA were also low in BMM and, moreover, whether like c-*myc* they were elevated in response to M-CSF. To investigate this the levels of *max* mRNA were analysed in both M-CSF-stimulated and unstimulated BMM and also in the macrophage cell line RAW. This revealed that not only was *max* mRNA expressed at detectable levels in both BMM and RAW, but its abundance was also elevated by treatment with M-CSF (Figure 14).

### 6.6 *Analysis of the changes in the levels of mad and mxi1 mRNAs during induced differentiation of HL60 cells.*

During the analysis of the levels of *max* mRNA during HL60 differentiation two other proteins were discovered that could form heterodimeric complexes with the Max protein, Mad (Ayer et al. 1993) and Mxi1 (Zervos et al. 1993). As described in the Introduction (section 1.4), the Mad:Max and Mxi1:Max complexes were found to be similar to the Myc:Max complex, in that they also bind the E-box motif (CACGTG). However, when they were assessed for their transactivation potential of this site, they appeared to differ in activity from Myc:Max and are thought to function as transcriptional repressors (Ayer et al.

Figure 14

Figure 14: *Analysis of the expression of max mRNA in bone marrow derived murine macrophages.*

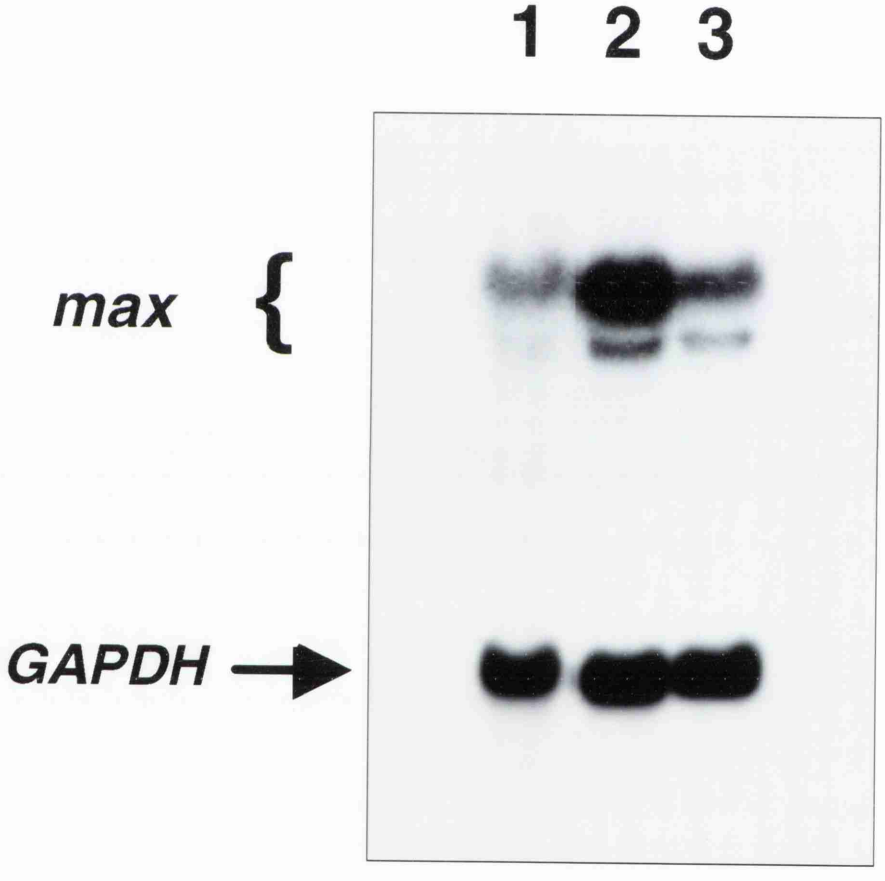
Northern blot analysis of RNAs isolated from murine bone-marrow-derived macrophages and RAW cells. Samples were obtained as follows:

1) Macrophages in standard culture (~10% cycling).

2) Cycling macrophages (stimulated for 24hr with M-CSF.).

3) RAW cells.

The blot was sequentially hybridised with probes for *max* and *GAPDH* and sections are labelled accordingly. RNA samples were kindly provided by Alan Reid of the Beatson Institute; for details of the growth conditions and treatment of bone marrow-derived macrophages see Reid (1992).


1 2 3
max
GAPDH

1993; Zervos et al. 1993). Therefore as either of these proteins is considered to be in competition with Myc for both available Max protein and available E-box sites, it is reasonable to assume that any increase in their abundances could well have a bearing on c-Myc function. With this in mind an analysis was made for changes in the levels of *mad* and *mxi1* mRNAs during induced differentiation of HL60 cells.

Re-probing of the blots used in Figure 11 with a probe for *mxi1* resulted, in agreement with other reports (Zervos et al. 1993; Larsson et al. 1994), in hybridisation to an RNA species of approximately 3kb. When the levels of this RNA were monitored during differentiation to monocytes/macrophages, it was found that its abundance remained largely invariant during the whole of the differentiation programme (Figure 15). In a similar manner, when *mxi1* levels were analysed during differentiation to granulocytes induced by DMSO, it was found that the abundance of the message remained stable for the majority of the time course, but was moderately decreased at the terminal stages of the differentiation programme (Figure 15).

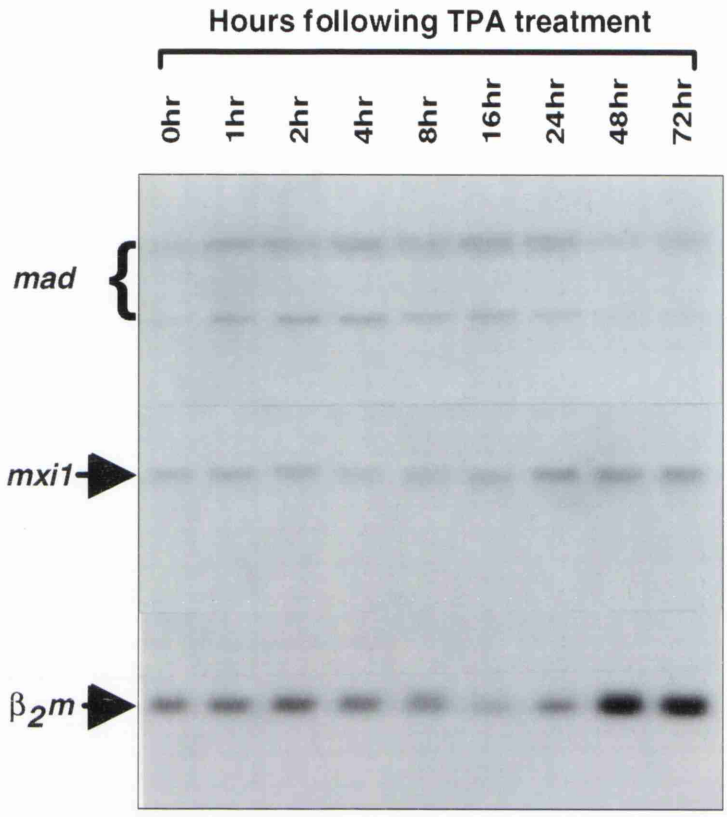
In contrast to the findings for *mxi1*, when analysis was made of *mad*-hybridising RNAs, a more interesting pattern of expression was revealed. Most markedly, during TPA-induced differentiation to monocytes/macrophages, it was found that the levels of *mad* undergo a small, but reproducible increase (2-3 fold as determined by densitometry) over the first hour of treatment with the differentiation inducer. Both the abundance of the 4kb and 7kb bands were increased over this time period and were then maintained at this elevated level until 24hr after the induction of the differentiation programme. After this time the abundances of both the RNAs were reduced again to the levels observed in uninduced cells.

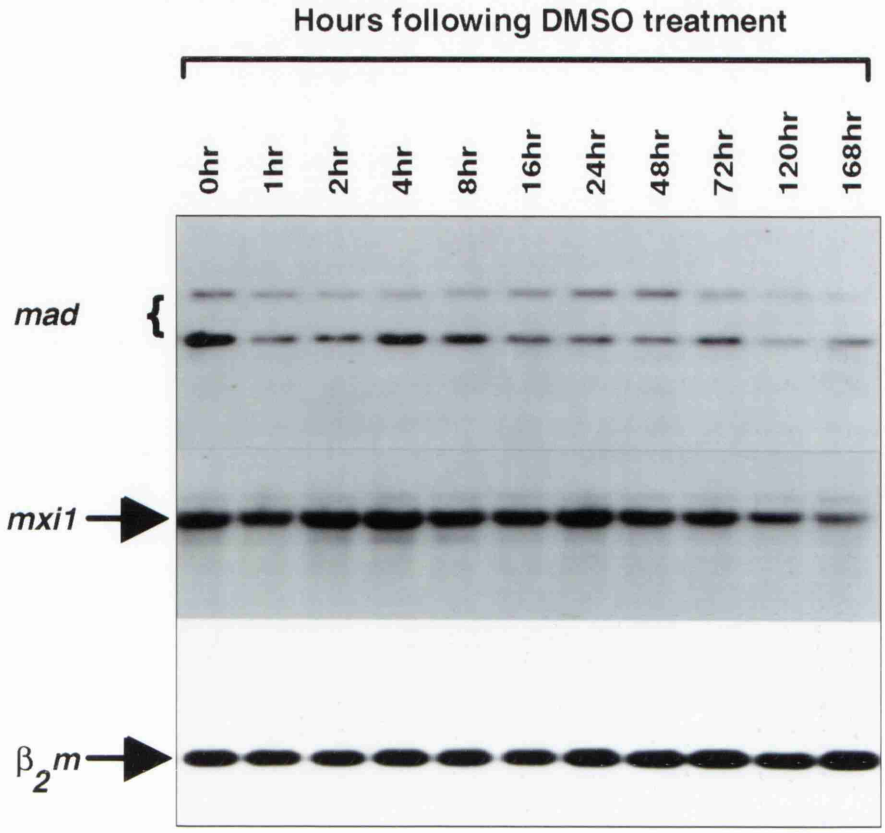
When the abundance of *mad*-hybridising RNAs were monitored during differentiation of HL60 cells to granulocytes, no increase in abundance was observed during the first hour of the differentiation programme. In addition, as the

Figure 15

Figure 15: *Northern analysis of the levels of mxi1 and mad mRNAs during differentiation of HL60 cells induced by DMSO and TPA.*

Northern blot analysis of RNAs isolated from HL60 cells which had been treated with either TPA (A) at a final concentration of $1.6 \times 10^{-7}$M or DMSO at a final concentration of 1.5% (v/v). RNA was isolated at the times indicated following addition of the differentiation inducer. The blots were sequentially hybridised with probes for *mad*, *mxi1* and $\beta_2$–*microglobulin* and sections are labelled accordingly.


Hours following TPA treatment
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
mad
mxi1
β2m


Hours following DMSO treatment
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
120hr
168hr
mad
mxi1
β2m

time course progressed, the levels of the RNAs appeared to fluctuate, particularly in the case of the smaller RNA, in a manner that was not observed during the differentiation of the cells to monocytes/macrophages.

### 6.7 *Analysis of the changes in the levels of c-myc, max, mad and mxi1 mRNAs during differentiation to monocytes/macrophages in the U937 cell line.*

In order to investigate whether any of any of the changes in mRNA abundances that were observed during differentiation in HL60 cells were truly associated with the differentiation process and not just a phenomenon of the HL60 cell line, it was decided to analyse the levels of these mRNAs in a second leukaemic cell line. As the most interesting changes, with respect to the early stages of differentiation, were observed when HL60 cells were differentiated to monocytes/macrophages (Figures 11 & 15), it was decided to analyse U937 cells as they can also be induced to differentiate into monocytes/macrophages in response to treatment with TPA (Harris and Ralph 1985).

Although U937 cells require 7 days of incubation with TPA, as compared to 3 days for HL60 (Figure 9), in order to achieve maximum differentiation, the cells undergo similar changes during the differentiation process. For example, they form large aggregates which adhere to the plastic of the tissue culture dish and in the latter stages of the differentiation programme exhibit very strong staining for the terminal differentiation marker, NSE (Figure 16).

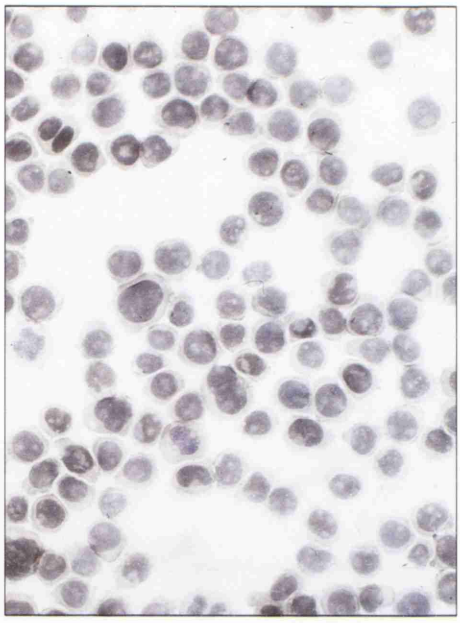
When the relative abundances of c-*myc*, *max*, *mad* and *mxi1* mRNAs were monitored during the induced differentiation of these cells very different patterns of change were observed from that seen following TPA treatment of HL60 cells (Figures 11 & 15). In fact, only the expression profile of *mxi1* was identical to that in HL60 cells, with the levels of the mRNA remaining largely invariant during the whole of the differentiation programme (Figure 17). In contrast, analysis of the

Figure 16

Figure 16: *Analysis of the terminal differentiation marker, non-specific esterase, in the U937 cell line following treatment with TPA.*

U937 cells were incubated either in the absence or presence of $1.6 \times 10^{-7}$M TPA for a period of 120hr. The cells were then stained for the activity of the terminal differentiation marker, non-specific esterase (see materials and methods). Sections of the figure are labelled accordingly.

Untreated
U937 cells

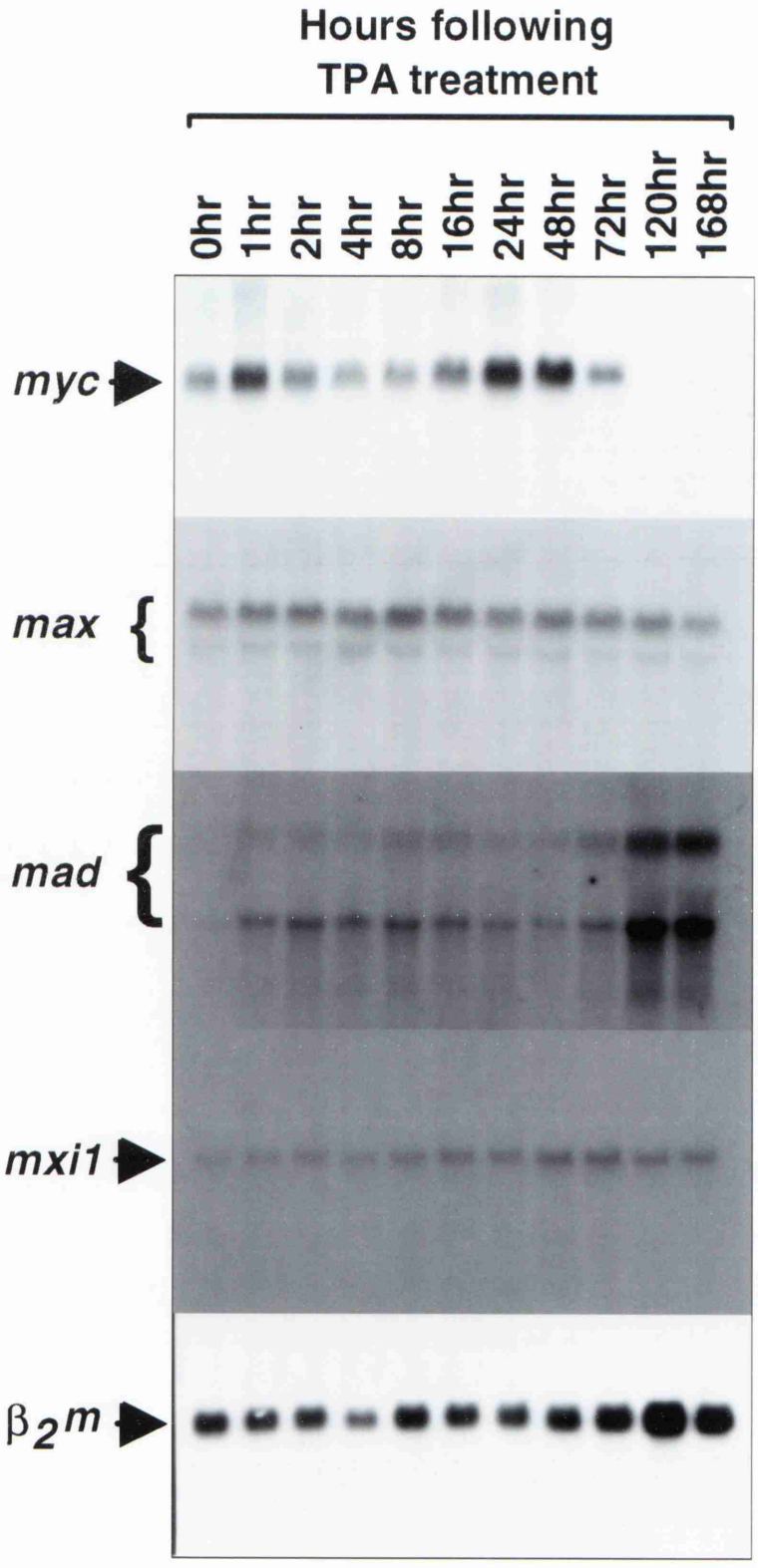


U937 cells
+120 hr TPA

Figure 17

Figure 17: *Northern analysis of the levels of c-myc, max, mad and mxi1 mRNAs during differentiation of U937 cells to monocytes/macrophages.*

Northern blot analysis of RNAs isolated from U937 cells which had been treated with TPA at a final concentration of $1.6 \times 10^{-7}$M. RNA was isolated at the times indicated following addition of the differentiation inducer. The blots were sequentially hybridised with probes for *mad*, *mxi1*, *max*, c-*myc* and $\beta_2$–*microglobulin* and sections are labelled accordingly.


Hours following
TPA treatment
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
120hr
168hr
myc
max
mad
mxi1
β2m

levels of c-*myc* mRNA revealed that, the abundance of the message was not rapidly down-regulated, as had been seen in HL60 (Figure 11), but was maintained at least at pre-induction levels until 72hr after treatment with the differentiation inducer. However, after this time point the abundance of the message was decreased to undetectable levels (Figure 17). Although in agreement with the study in HL60 cells (Figure 11), the levels of *max* mRNA appeared to be moderately decreased in the very late stages of the differentiation time course (168hr), the abundance of the message prior to this time point did not appear to fluctuate as was seen in during HL60 differentiation and no peaks of expression were observed (Figure 17). Analysis of the pattern of change of *mad*-hybridising RNAs revealed that, in a similar manner to what was seen in HL60 cells induced to differentiation by treatment with TPA (Figure 15), there was an increase in abundance of the both the 4kb and 7kb RNA species during the first hour of the differentiation programme. However, in contrast to what was observed in HL60 cells, the levels of the mRNAs did not return to pre-induction levels and were in fact super-induced between 72hr and 120hr after the addition of the differentiation inducer (Figure 17).

6.8 *Analysis of the expression of c-myc, max, mad and mxi1 in HL60 cells induced to differentiate to monocytes/macrophages by treatment with the vitamin D3 derivative, 1-α,25-dihydroxyvitamin D3 (Vit D3).*

As described above, it was found that the levels of *mad* mRNA are induced prior to the down-regulation of c-*myc* during monocyte/macrophage differentiation in both HL60 (Figure 15) and U937 cells (Figure 16). This led to the speculation that the abrogation of the differentiation-blocking activity of c-*myc* might not primarily occur through the loss of c-Myc, but through an increase in the levels of the c-Myc antagonist, Mad. In addition, since we also found that an increase in *mad* levels were not required for differentiation of HL60 cells to

granulocytes (Figure 15), we were interested to know whether the increase was an essential event during differentiation to monocytes/macrophages and therefore a possible determinator of this lineage. To analyse if differentiation to monocytes/macrophages could be achieved without an increase in the levels of *mad* mRNA, we treated HL60 cells with a different inducer of this differentiation pathway, 1-α,25-dihydroxyvitamin D3 (Vit D3) (McCarthy et al. 1983). Following treatment with this inducer, it was found that, in a similar manner to that which we observed during induction with DMSO (Figure 11), the cells could be differentiated (Figure 9), without an accompanying increase in the abundance of *mad* mRNA (Figure 18). Therefore, while the importance of the induction of *mad* expression during differentiation of HL60 to monocytes/macrophages following treatment with TPA cannot be discounted, the ability of the cells to undergo a programme of differentiation to either granulocytes or monocytes/macrophages is not dependent on an increase in the levels of *mad* mRNA.

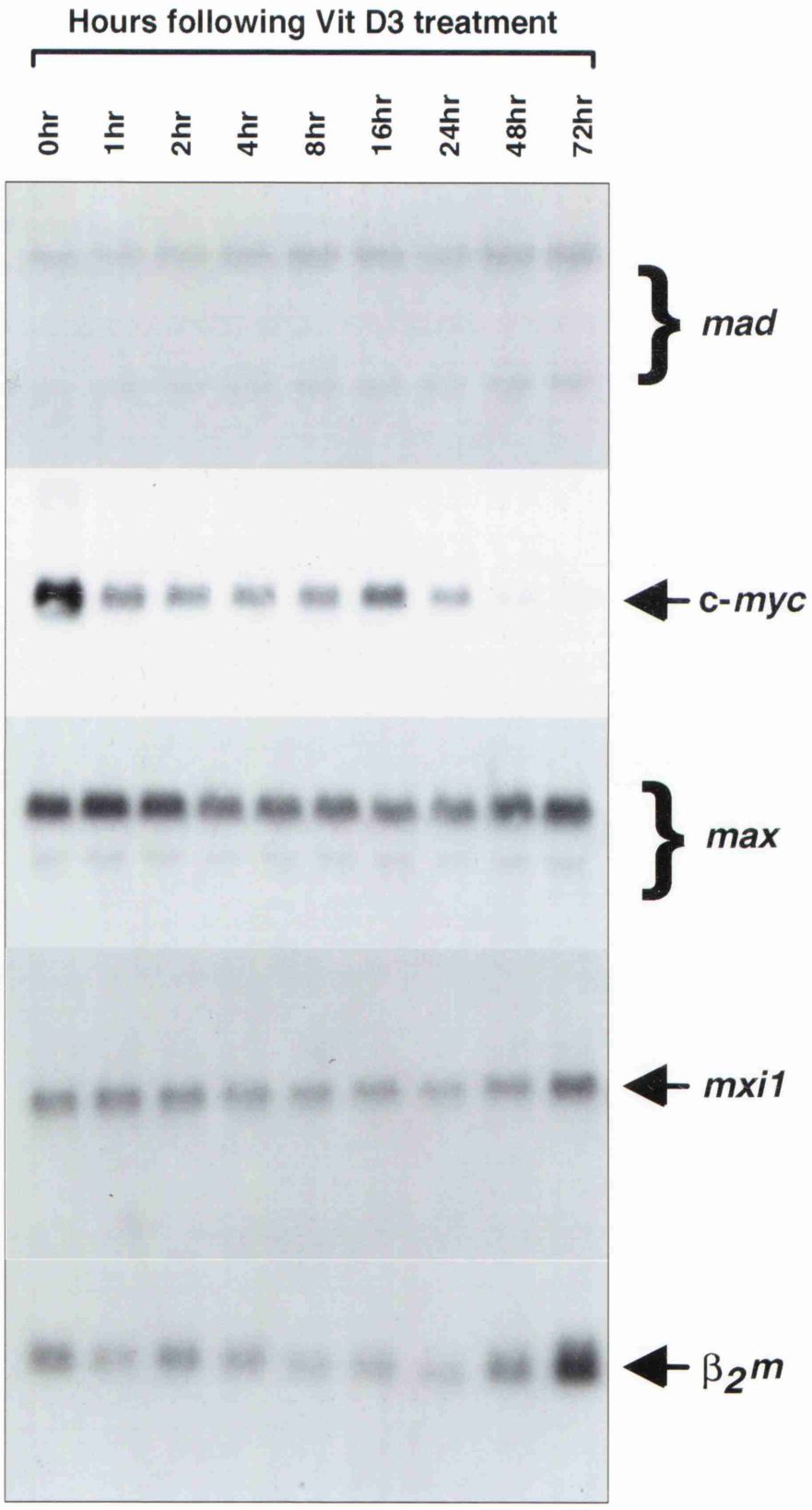
Analysis of the other mRNAs following treatment with Vit D3 revealed once again that the levels of *mxi1* are unaffected by differentiation induction in HL60 cells (Figure 18). In contrast, the levels of *max* mRNA were more similar to what was observed during TPA-induced differentiation in U937 (Figure 17) than those observed during differentiation in HL60 (Figure 11). For the majority of the differentiation programme, the abundance of the mRNA remained largely invariant and, if the loading control is to be taken into account, was only moderately decreased between 48hr and 72hr after the addition of the Vit D3 (Figure 18).

Analysis of the changes in the levels of c-*myc* mRNA revealed a profile of expression that was not only different from that which was observed during differentiation in HL60 cells (Figure 11), but which was also different form that observed during differentiation in U937 (Figure 17). As can be seen in Figure

Figure 18

Figure 18: *Northern analysis of the levels of c-myc, max, mad and mxi1 mRNAs during differentiation of HL60 cells following treatment with Vit D3.*

RNAs were isolated, at the times indicated, from HL60 cells during monocytic differentiation induced by 1-α,25-dihyroxyvitamin D3 at a final concentration of 1 x $10^{-7}$M. The blot was hybridised sequentially with probes for *mad*, *mxi1*, *max*, c-*myc* and $\beta_2$-*microglobulin*. Sections of the figure corresponding to each probing are indicated.


Hours following Vit D3 treatment
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
mad
c-myc
max
mxi1
β2m

18, the mRNA appeared to undergo a biphasic decrease in abundance following treatment with the differentiation inducer. After an initial decrease after only 1hr of treatment with Vit D3, the abundance of the mRNA was then maintained at an intermediate level before being decreased again between 24hr and 48hr after the induction of the differentiation programme.

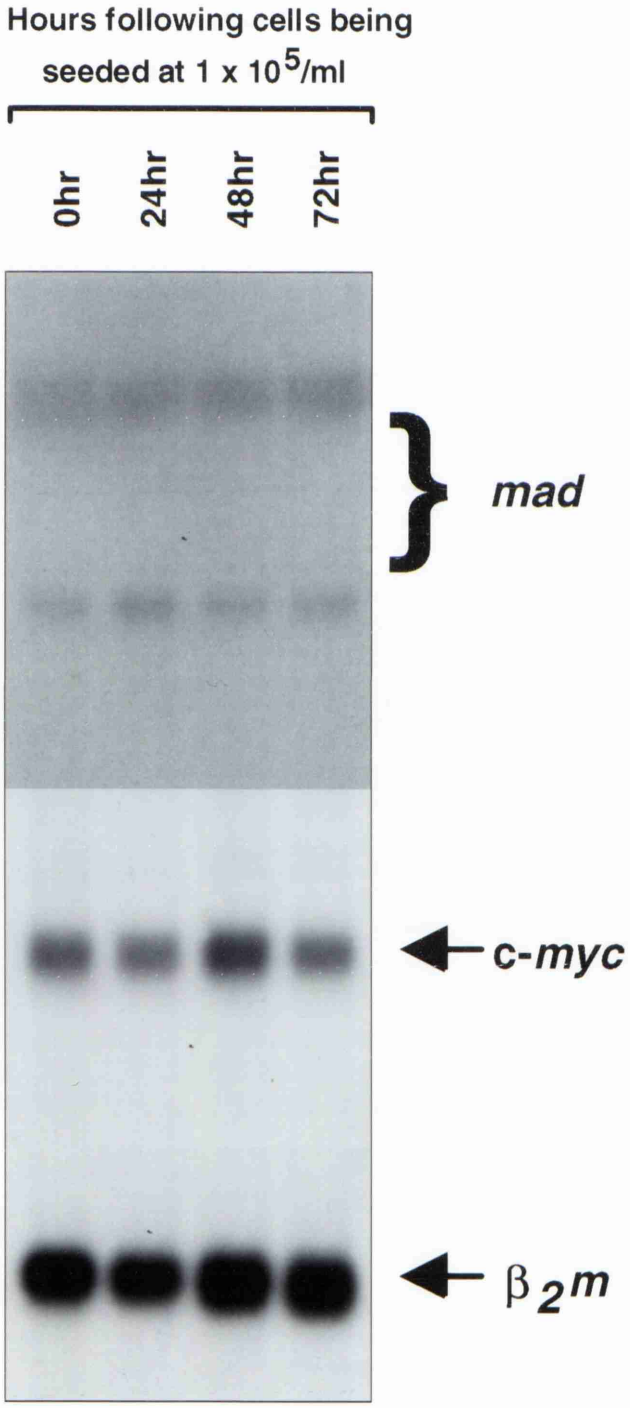
It was initially considered that this second decrease in c-*myc* mRNA could be as a result of natural fluctuations in the levels of the mRNA that occur prior to the time at which the cells are normally due to be passaged. As a result the abundance of c-*myc* was monitored in HL60 cells which were handled in exactly the same way as cells used in differentiation studies, but which were left untreated and maintained in culture, without passage for a period of 72hr. Northern analysis of the levels of c-*myc* mRNA in these cells revealed that, unlike what was seen following treatment of HL60 with Vit D3, the abundance of c-*myc* was not down-regulated after 24hr of time in culture (Figure 19). Moreover, the levels of c-*myc* mRNA did not at any point decrease below that observed at the 0hr time point. This therefore shows that the second decrease of c-*myc* mRNA that was observed in Figure 18, was indeed a result of treating the cells with Vit D3 and was not merely a change that occurs during the standard culture of HL60 cells.

The speculations about the second stage of c-*myc* down-regulation following treatment with Vit D3 resulted in consideration that the decrease in the levels of *mad* mRNA that occur in the latter stages of the TPA-induced differentiation in HL60 cells (Figure 15) could also be affected in a similar manner. However, when this was assessed, it was found that in a similar manner to c-*myc*, the abundance of *mad* mRNA was also unaffected by simply maintaining cells in culture for a period of 3 days (Figure 19).

Figure 19

Figure 19: *Analysis of the levels of c-myc and mad mRNAs in untreated HL60 cells left in culture for a period of 3 days.*

Northern blot analysis of RNAs isolated from untreated HL60 cells which were treated in exactly the same manner as those used in differentiation studies. The blot was hybridised sequentially with probes for *mad*, *mxi1*, *max*, c-*myc* and $\beta_2$-*microglobulin.* Sections of the figure corresponding to each probing are indicated.


Hours following cells being seeded at 1 x 10^5/ml
0hr
24hr
48hr
72hr
mad
c-myc
β2m

6.9 *Analysis of the profiles of change in mRNA abundance of c-myc, max, mad and mxi1 following TPA treatment of the differentiation-defective variant of the HL60 cell line, HL60-Ast4.*

The ability to make a specific association between a change in mRNA abundance and the establishment of the differentiated phenotype is complicated in leukaemic cell lines due to the concomitant growth arrest that these cells undergo following induction of the differentiation programme. In order to try to circumvent this problem, we analysed the levels of c-*myc*, *max* , *mad* and *mxi1* mRNAs in a variant of the HL60 cell line which differs in its response to treatment with the differentiation inducer. This variant, HL60-Ast4, is different from the parental HL60 cell line in that in does not differentiate to monocytes/macrophages following incubation with TPA at a final concentration of $1.6 \times 10^{-7}$M (Bunce et al. 1983). In addition, not only does this line not show positive staining for NSE activity (Figure 20), it does not form aggregates and remains as single, unattached cells in the culture medium. However, in spite of this line's inability to differentiate, it still responds to the TPA treatment by undergoing a growth arrest that is not observed when cells are incubated for the same period of time without the addition of the phorbol ester (Figure 21). Moreover, following this growth arrest the cells rapidly undergo a programme of cell death and are therefore, unfortunately, similar to "wild-type" HL60 cells in that they cannot be analysed beyond a period of 72-96hr after the addition of the phorbol ester.

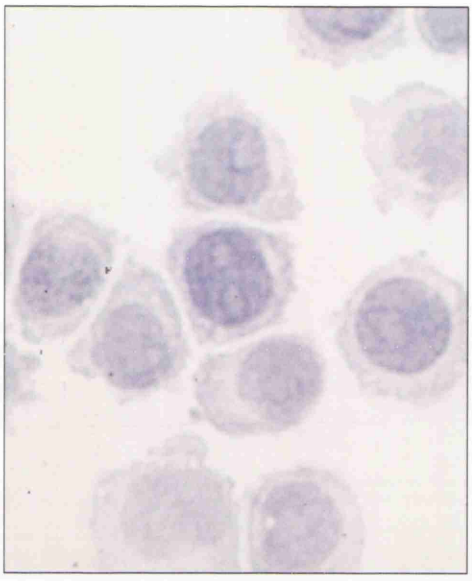
Due to the phenotype of this line, it was reasonable to consider that any change in the levels of an mRNA that was seen during "wild-type" HL60 (wt-HL60) differentiation and that was specifically associated with the differentiation process *per se*, should not be seen following the TPA treatment of HL60-Ast4. However, when analysis was made of the patterns of change of *mad*

Figure 20

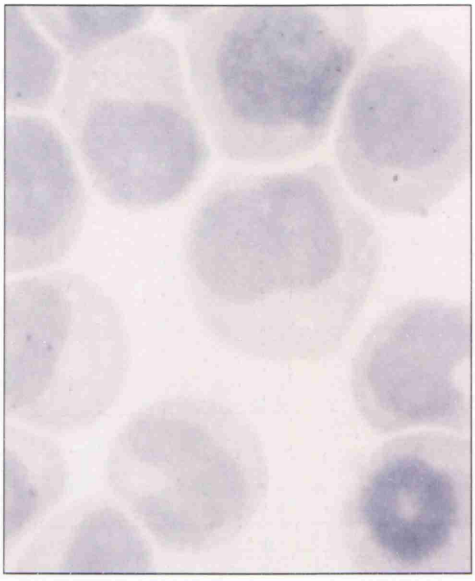
Figure 20: *Comparison of the differentiation capacity of HL60 and HL60-Ast4 cells following treatment with TPA.*

HL60 and HL60-Ast4 cells were treated for a period of 3 days with TPA at a final concentration of $1.6 \times 10^{-7}$M. Uninduced cells were incubated for the same period of time with an equal amount of solvent that was used in the phorbol ester treatments. Cells were stained for the activity of the terminal differentiation marker, non-specific esterase. Photographs of cells were taken at x40 magnification, except for the picture of HL60 + TPA which, due to the formation of large cell aggregates, was taken at x10.

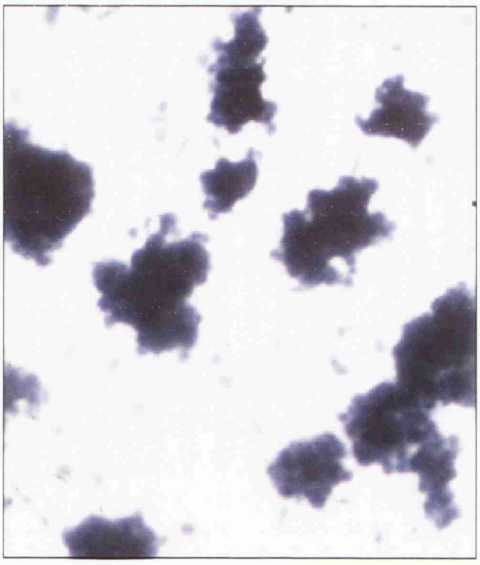
HL60
Uninduced



Ast4
Uninduced



HL60
+TPA



Ast4
+TPA

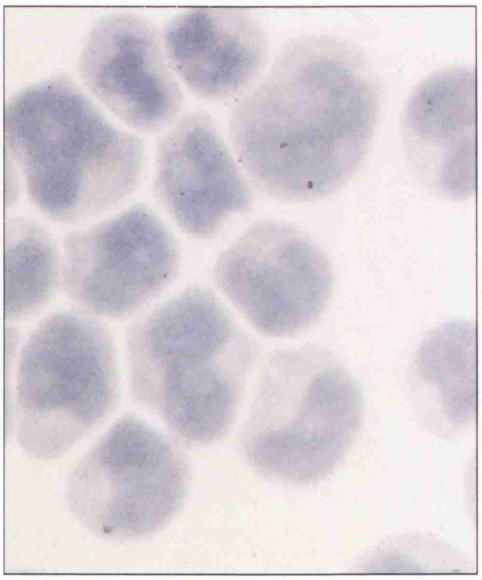
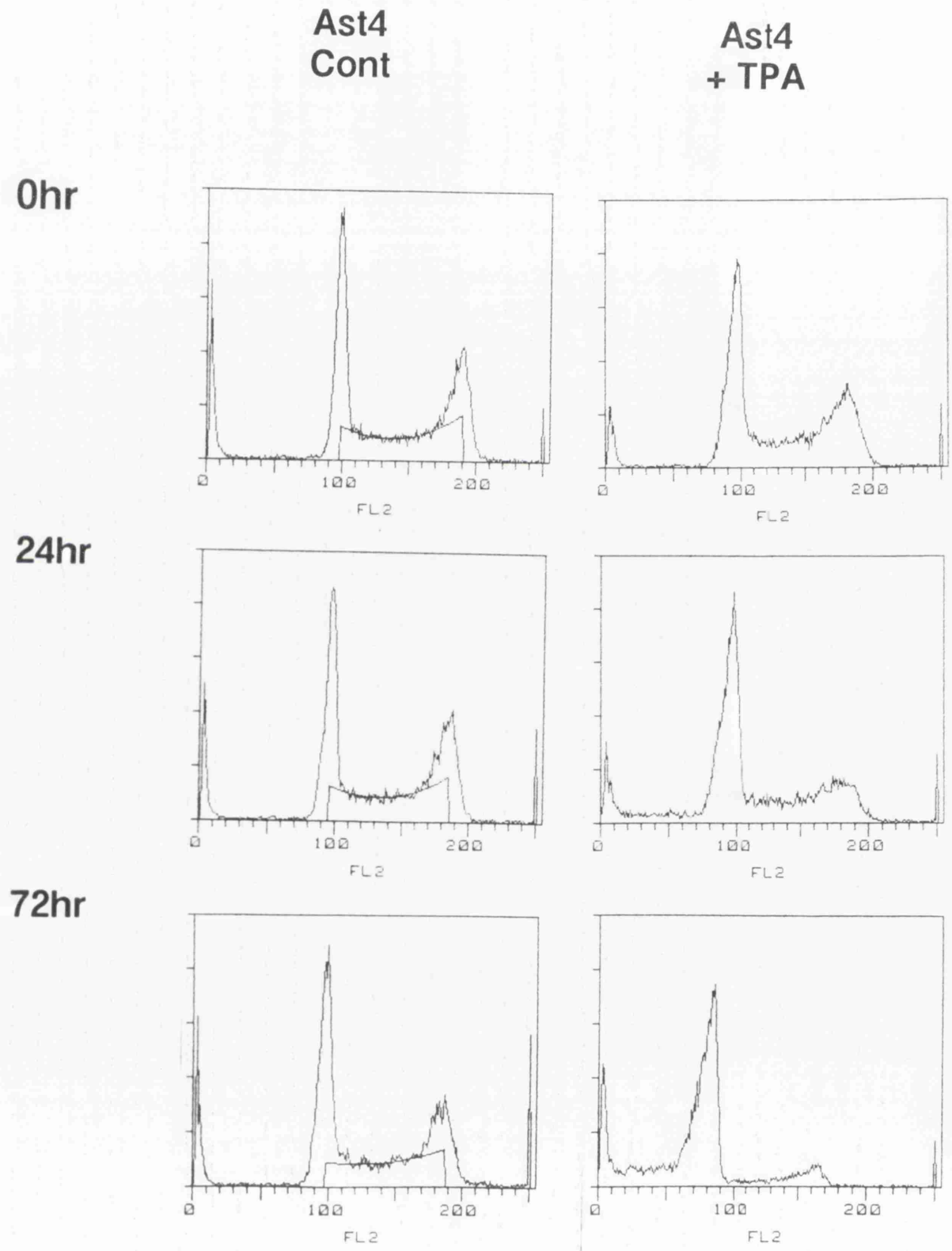


Figure 21

Figure 21: *Analysis of the effects of TPA-treatment on the cell-cycle status of HL60 and HL60-Ast4 cells.*

Analysis by flow cytometry of the DNA content of populations of HL60-Ast4 cells either maintained for 3 days in the presence or absence of TPA at a final concentration of $1.6 \times 10^{-7}$M. The x axis of the profiles represents the fluorescence activity that is proportional to the DNA content of cells within the sample analysed. The y axis represents the number of cells. Equal numbers of events (10,000) were analysed for each of the time points indicated. The peak in the extreme left of each profile represents chicken reticulocytes which were added to each sample as a reference point. A second line is present on the profiles of the untreated cells, which was added by the computer during integration calculations. This line is not shown on the profiles of treated cells.


Ast4
Cont
Ast4
+ TPA
0hr
24hr
72hr
0
100
200
FL2

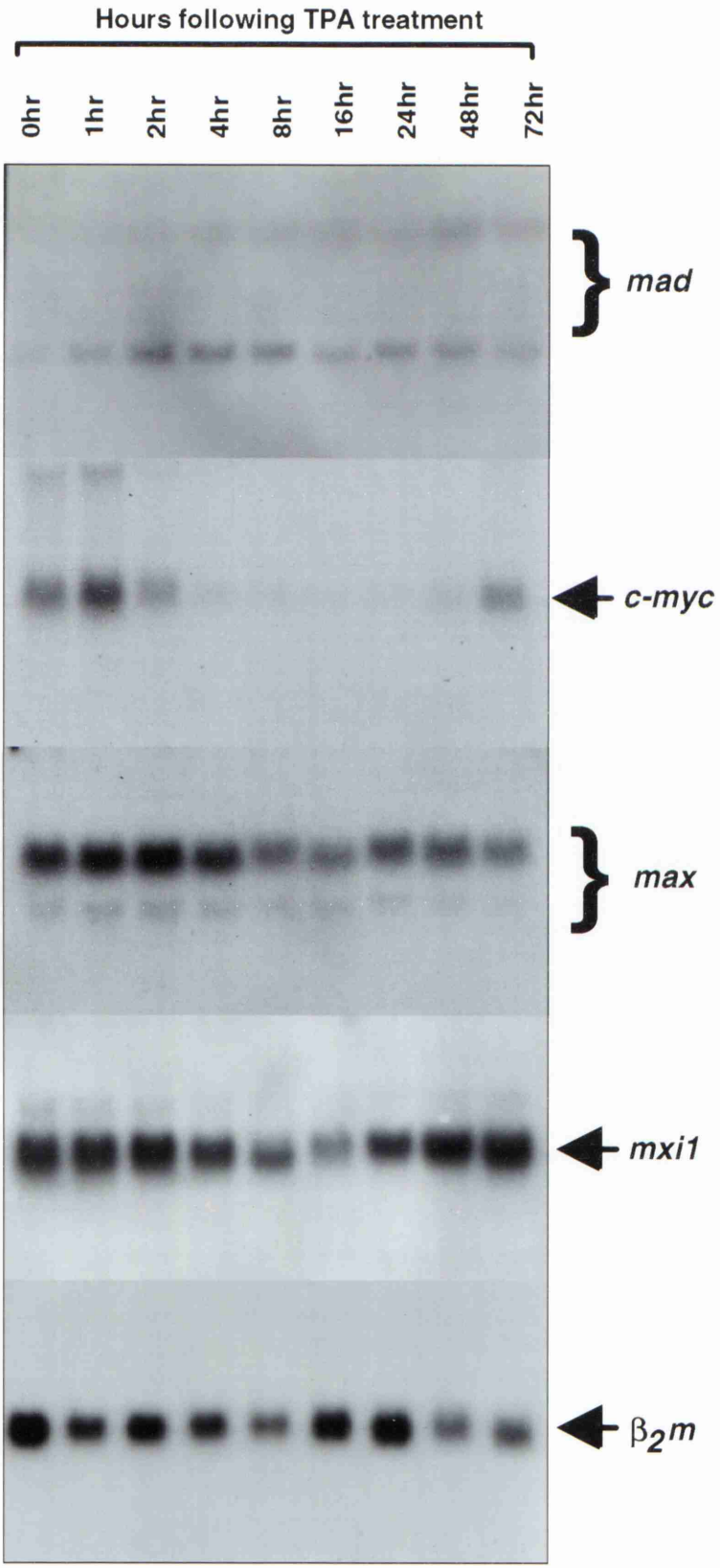
mRNAs in Ast4 very few differences were observed. In particular, it was found that the abundance of *mad* mRNA, in an essentially identical manner to what was observed in wt-HL60 cells (Figure 15), was increased over the first hour of TPA treatment (Figure 22). However, in contrast to wt-HL60 cells, these levels were then maintained for the remainder of the time course and were not decreased to pre-induction levels after 24hr of TPA treatment. Although on the basis of the initial assumption, this finding lends support to the notion that up-regulation of *mad* mRNA is not a determining event within this lineage, it can in no way completely exclude this possibility. For example, it is possible that the mutation which renders Ast4 cells unable to differentiate lies downstream of signals which results from increased levels of Mad. Therefore, while in this scenario it is feasible that an increase in the abundance of *mad* mRNA in wt-HL60 cells could invoke a programme of differentiation, no such effect would be seen in Ast4 cells. Consequently, although this finding is indeed very intriguing, elucidation of the role of *mad* in these cells certainly requires and also merits additional investigation.

When the levels of *mxi1* mRNA were monitored following TPA treatment of Ast4, it was again found that the pattern of change of the RNA was very similar to what was seen following treatment of "wild-type" cells. Despite a small decrease in the abundance of the message after approximately 8hr-16hr after addition of the TPA, the levels of the mRNA remained largely invariant for the whole of the period examined (Figure 22). In a similar manner, analysis of the levels of *max* mRNA also revealed a pattern of change that was not outstandingly different from the pattern observed in wt-HL60. After an initial transient increase in the abundance of the message after approximately 1-2hrs of treatment with the phorbol ester, the levels of the mRNA then underwent moderate fluctuations for the remainder of the times analysed (Figure 22). However, in contrast to what was observed during TPA-induced differentiation in HL60 cells (Figure 11), the levels of *max* mRNA did not undergo a marked decrease between 48hr and 72hr after the

Figure 22

Figure 22: *Northern analysis of the levels of c-myc, max, mad and mxi1 mRNAs following treatment of HL60-Ast4 cells with TPA.*

Northern blot analysis of RNAs isolated from HL60-Ast4 cells which had been treated with TPA (A) at a final concentration of $1.6 \times 10^{-7}$M. RNA was isolated at the times indicated following addition of the differentiation inducer. The blots were sequentially hybridised with probes for *mad*, *mxi1*, *max*, c-*myc* and $\beta_2$–*microglobulin* and sections are labelled accordingly.


Hours following TPA treatment
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
mad
c-myc
max
mxi1
β2m

addition of the phorbol ester. However, how this difference relates to differentiation in wt-HL60 cells remains to be determined.

Undoubtedly the most striking difference between the changes in mRNA abundance in HL60 and HL60-Ast4 cells, was that which was observed for the c-*myc* message. Although in the early stages of treatment (1-4hr) the profiles were indeed very similar, the levels apparent in the latter stages were very different (Figure 22). More precisely, while in wt-HL60 the levels remain low after their down-regulation following the 2hr time point (Figure 11), the levels in HL60-Ast4 were re-established after 72hr of treatment to the those which were evident in uninduced cells.

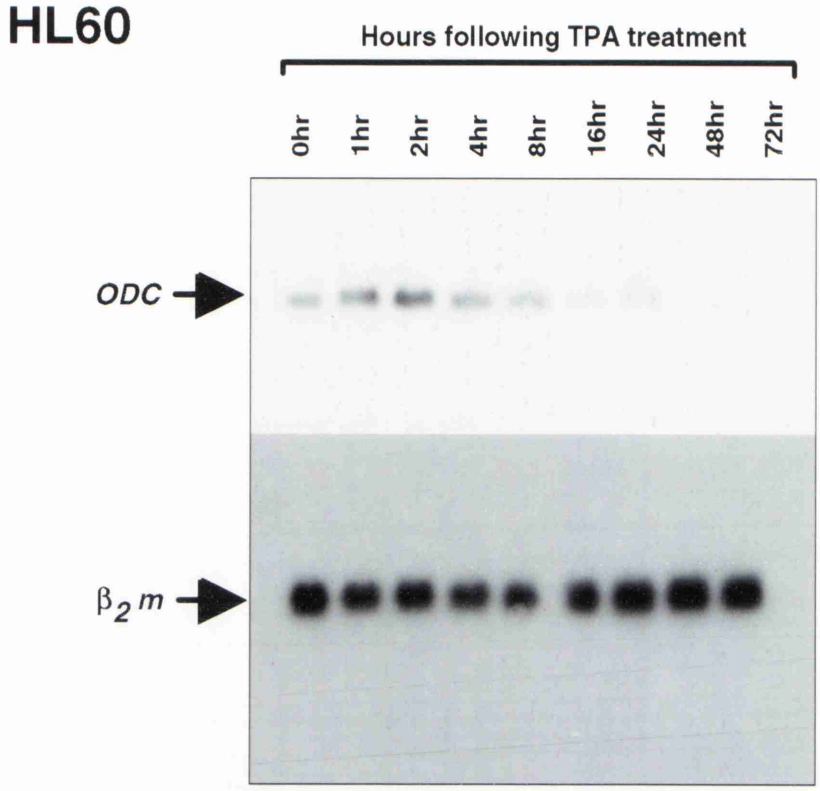
### 6.10 *Analysis of the levels of ODC mRNA following TPA treatment of "wild-type" HL60 cells and HL60-Ast4.*

It has been shown that c-Myc can up-regulate the expression of the mRNA for ornithine decarboxylase (ODC) (Wagner et al. 1993). In addition, studies have indicated that the activity of ODC is essential for c-Myc's ability to cause cell-cycle progression and apoptosis (Packham and Cleveland 1994). Therefore, intrigued by the finding that the levels of c-*myc* mRNA are re-established in Ast4 after 72hr of treatment with TPA (Figure 22), it was decided to analyse if this increase in c-*myc* was translated into an increase in the levels of *ODC* mRNA. To this end, northern blots of RNA isolated from HL60 and HL60-Ast4 following treatment with TPA were hybridised with a cDNA probe corresponding to the coding region of the human *ODC* mRNA (see Methods 5.5.1). This revealed, in agreement with previous studies (Rius and Aller 1989), that the abundance of *ODC* mRNA is decreased during TPA-induced differentiation in HL60 cells (Figure 23). However, when analysis was made of *ODC* mRNA following TPA treatment of Ast4 cells it was a surprise to find that not only were the levels of *ODC* message not

Figure 23

Figure 23: *Northern analysis of the levels of ODC mRNA in HL60 and HL60-Ast4 cells following treatment with TPA.*

Northern blot analysis of RNAs isolated, at the times indicated, from HL60 and HL60-Ast4 cells following treatment with TPA at a final concentration of $1.6 \times 10^{-7}$M. The blots was initially hybridised with a cDNA probe for *ODC*, subsequently stripped and then as an assessment of loading were re-hybridised with a probe for $\beta_2$–*microglobulin.* Sections of the figure relating to each probing are labelled accordingly.


HL60
Hours following TPA treatment
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
ODC
β2m

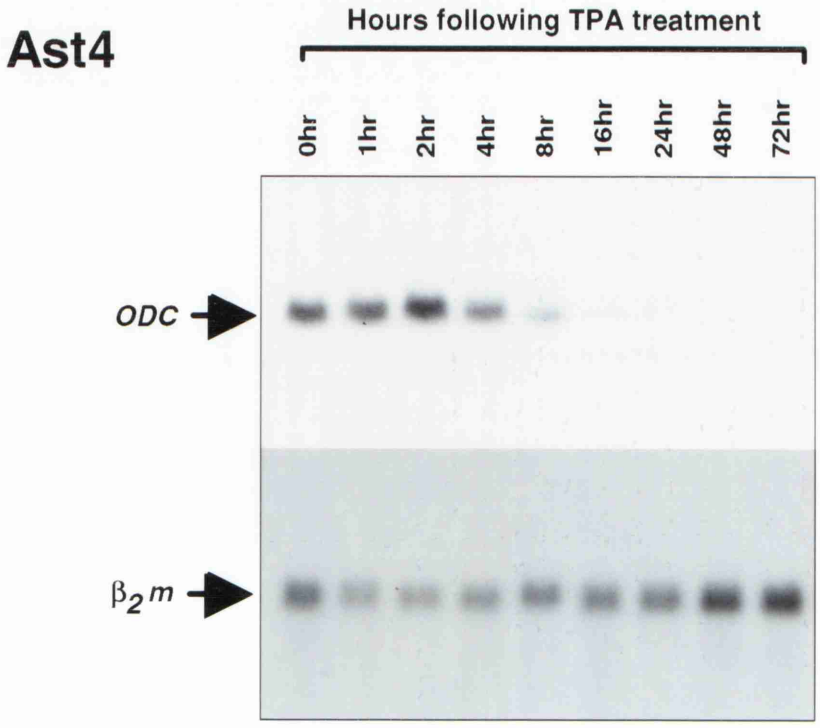
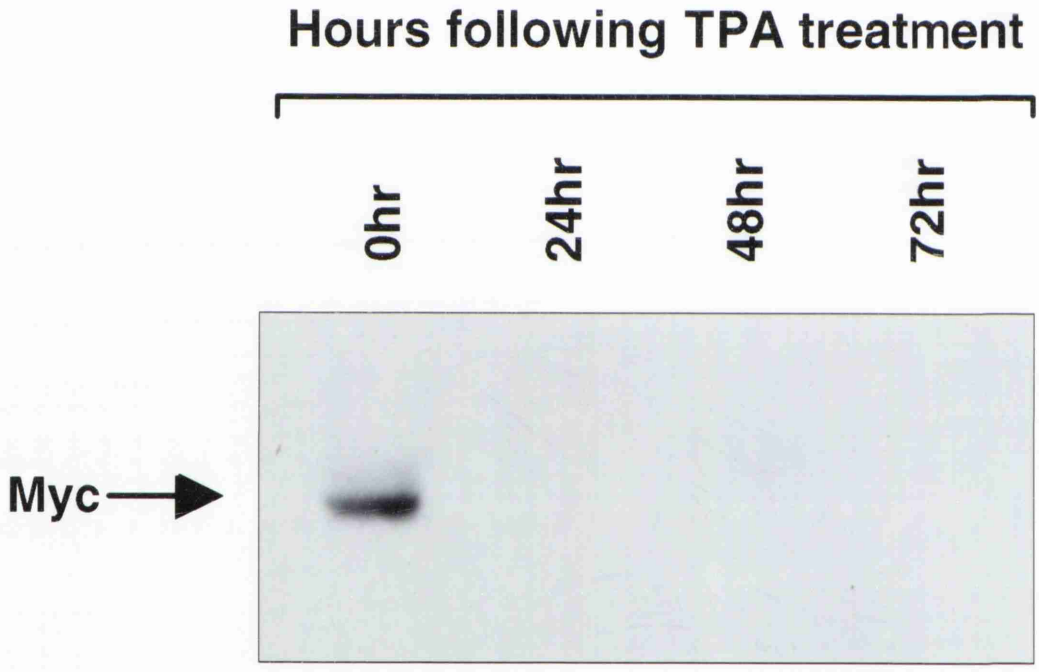

Ast4
Hours following TPA treatment
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
ODC
β2m

Figure 24

Figure 24: *Western blot analysis of the levels of c-Myc protein in HL60-Ast4 cells following treatment with TPA.*

Western blot analysis of the levels of c-Myc protein in HL60-Ast4 cells following treatment with TPA, for the times indicated, at a final concentration of $1 \times 10^{-7}$ M. Equal quantities of protein from each time point were resolved through polyacrylamide gels, blotted and assessed for Myc protein using an antiserum which had been raised against the full-length human c-Myc protein.


Hours following TPA treatment
0hr
24hr
48hr
72hr
Myc

re-established in the latter stages of the time course, but they appeared to be decreased more rapidly than in the "wild-type HL60 cells (Figure 23).

While no easy explanation could be found as to why the levels of *ODC* mRNA are decreased rapidly in HL60-Ast4 than in wt-HL60, it seemed possible that the abundance of the message was not re-established in the latter stages of the time course because, although the cells re-establish the levels of c-*myc* mRNA (Figure 22), this is not translated into increased levels of c-Myc protein. In order to ascertain if this were the case, protein samples from TPA-treated Ast4 cells were Western blotted and probed with the an antiserum raised against the full-length human c-Myc protein (see Methods 5.7.1). This indicated that although there were substantial amounts of c-Myc protein in untreated HL60-Ast4 cells, no c-Myc was detectable in the cells after treating them with TPA for either 24hr, 48hr or 72hr (Figure 24). This finding therefore provides a reason for why HL60-Ast4 cells do not re-establish the levels of *ODC* mRNA.

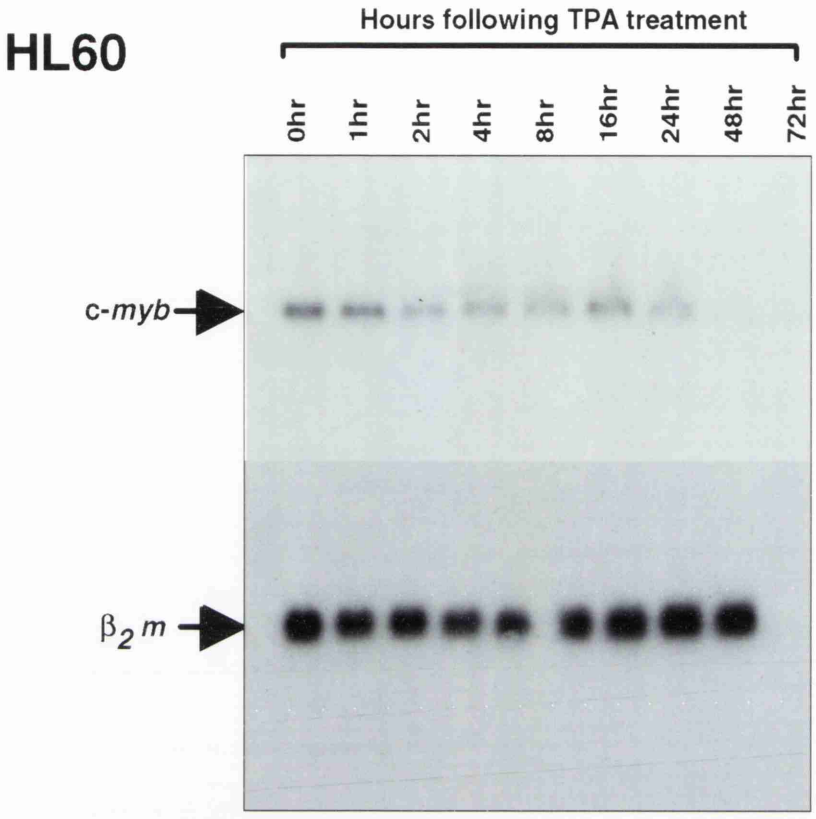
### 6.11 *Analysis of the patterns of change in c-myb mRNA abundance following TPA treatment of "wild type" HL60 and HL60-Ast4 cells.*

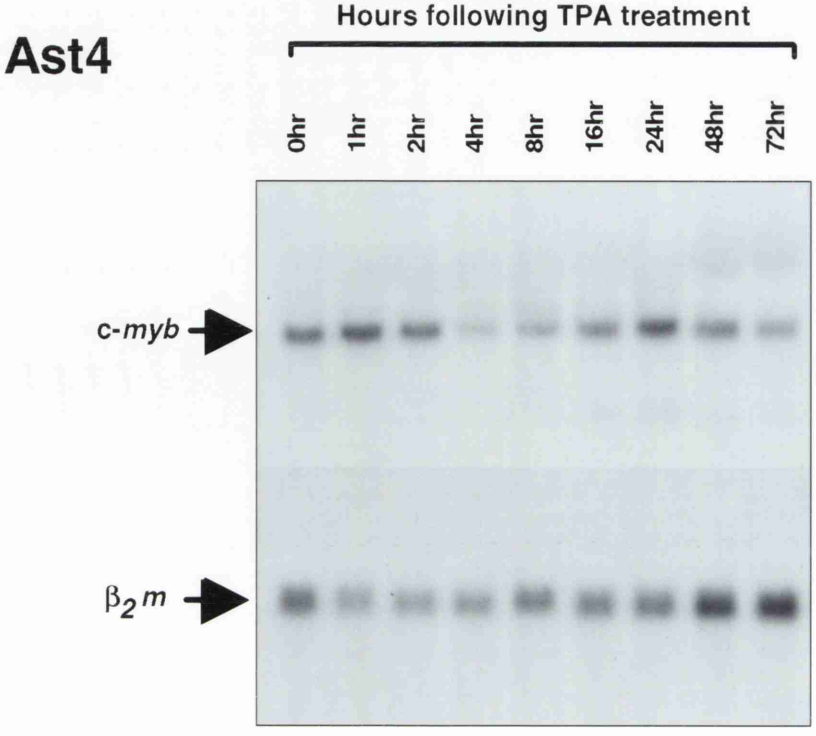
Studies have shown that constitutive expression of the c-*myb* proto-oncogene is able to block the differentiation of myeloid cells (Clarke et al. 1988). In addition, the protein product of the c-*myb* gene has been implicated in the control of transcription of c-*myc* (Nakagoshi et al. 1992). Therefore, it has become considered that it is possibly through the transactivation of c-*myc* that c-*myb*, at least in part, brings about these phenotypic effects. In light of this, it was decided to analyse the changes in the levels of c-*myb* mRNA following the TPA treatment of both "wild-type" HL60 and HL60-Ast4 cells. As can be seen in Figure 25, this showed that, in a agreement with previous studies (Boise et al. 1992) that the abundance of c-*myb* mRNA is decreased during TPA-induced differentiation of

Figure 25

Figure 25: *Northern analysis of the levels of c-myb mRNA in HL60 and HL60-Ast4 cells following treatment with TPA.*

Northern blot analysis of RNAs isolated, at the times indicated, from HL60 and HL60-Ast4 cells following treatment with TPA at a final concentration of $1.6 \times 10^{-7}$M. The blots was initially hybridised with a cDNA probe for c-*myb*, subsequently stripped and then as an assessment of loading were re-hybridised with a probe for $\beta_2$–*microglobulin.* Sections of the figure relating to each probing are labelled accordingly.


HL60
Hours following TPA treatment
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
c-myb
β2m


Ast4
Hours following TPA treatment
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
c-myb
β2m

HL60 cells. However, when analysis was made of the levels c-*myb* following TPA treatment of HL60-Ast4 cells, a very different pattern of change was observed. Although it was evident that the abundance of the mRNA was transiently decreased after approximately 4hrs after the addition of the phorbol ester, the levels were then rapidly re-established to pre-induction levels after 8hrs and then maintained at this level for the remainder of the period analysed (Figure 25). This finding therefore shows that c-*myb*, as well as c-*myc*, is regulated differently in HL60-Ast4 cells than it is in wt-HL60 cells. However, further experiments would be required to determine if this altered regulation of c-*myb* is indeed the reason for the re-establishment of the abundance of c-*myc* mRNA that was observed in Figure 22.

## 6.12 *Analysis of the effects of treatment with the diacylglycerol analogue, 1-oleoyl-2-acetyl-sn-glycerol (OAG), on the abundance of mad mRNA in U937 cells.*

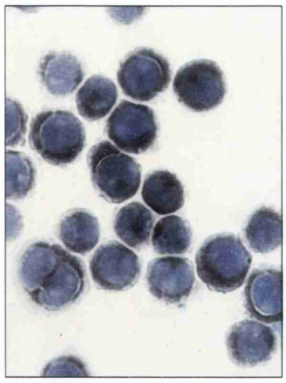
Previous experiments had shown that up-regulation of the levels of *mad* mRNA was not an essential event for differentiation to either granulocytes (Figure 15) or monocytes/macrophages (Figure 18) in myeloid leukaemic cells. In light of this, it was deemed interesting to determine if the abundance of *mad* mRNA could be increased without the cells undergoing an accompanying programme of differentiation. If this proved possible, this would therefore indicate that an increase in the levels of *mad* mRNA is neither sufficient nor obligatory for induced differentiation within these systems. To this end, an analysis was made of the literature regarding the mechanism of action of each of the differentiation inducers used. As a result, this revealed a large body of evidence which suggested that the phenotypic effects of TPA may be mediated through the activation of protein kinase C (Castagna et al. 1982; Evans and Hassan 1993). Moreover, it is

Figure 26

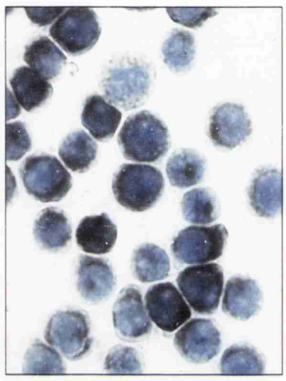
Figure 26: *Analysis of the effects of treating U937 cells with the diacylglycerol analogue OAG.*

The effect of OAG treatment on the differentiation status of U937 cells was assessed by staining for the activity of non-specific esterase. The activity of the marker in cells incubated in the absence and presence of OAG was compared to that in both untreated cells and cells differentiated by treatment with TPA.

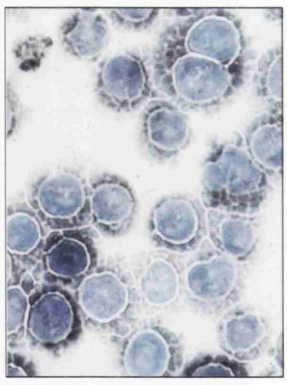
Untreated Cells



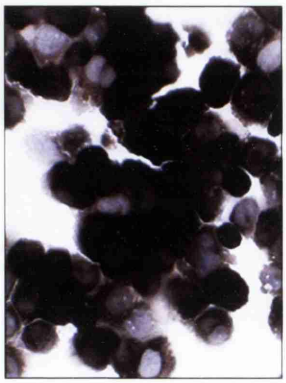
72hr OAG-



72hr OAG+



120hr +TPA



thought that TPA affects this enzyme by mimicking the effects of its endogenous activator, diacylglycerol (Nakaki et al. 1984; Sharkey et al. 1984).

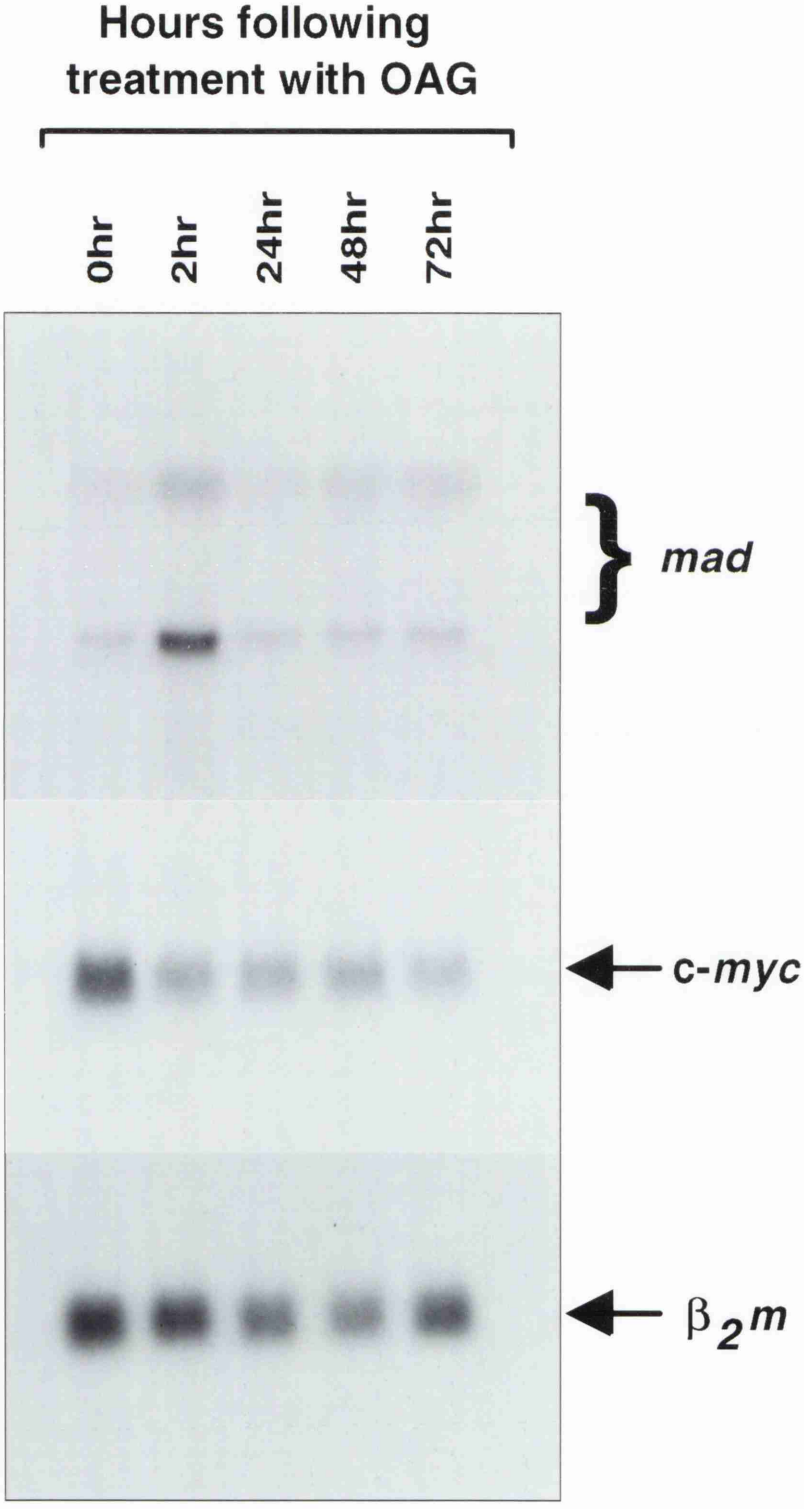
Therefore, in an attempt to imitate at least some of the effects of TPA, we treated U937 cells with a synthetic analogue of diacylglycerol, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) (Kaibuchi et al. 1983). When analysis was made of these cells, it was apparent that, in agreement with a previous report (Ways et al. 1987), OAG did not cause any differentiation-associated, morphological change in these cells. In addition, unlike treatment with TPA, it did not affect the activity of the terminal differentiation marker, NSE (Figure 26). Nevertheless, in repeated experiments, treatment with this compound seemed to affect the appearance of the outer membrane and cytoplasm of the cells (Figure 26). However, both the mechanism and consequence of this effect remain unknown.

In agreement with the initial hypothesis, it was found that the levels of *mad* mRNA are indeed increased as a result of treating U937 cells with OAG (Figure 27). However, this increase, which was observed after 2hr of OAG treatment, was only transient and the abundance of the message was returned to pre-induction levels prior to the next time point analysed (24hr). This therefore shows that an increase in the abundance of *mad* mRNA is not sufficient for the induction of differentiation in U937 cells. However, because the pattern of expression is not identical to that observed following treatment with TPA (Figure 17), it still remains possible that differentiation could well occur if the increased levels of *mad* mRNA were maintained for a much longer period of time. Further studies are therefore required to fully discover the roles played by *mad* mRNA and protein in the differentiation of these cells.

Figure 27

Figure 27: *Northern analysis of the levels of c-myc and mad mRNAs following treatment with the diacylglycerol analogue, OAG.*

Northern blot analysis of RNAs isolated from U937 cells which had been treated daily with OAG to give a final concentration of 20μg/ml. RNA was isolated at the times indicated following addition of the differentiation inducer. The blots were sequentially hybridised with probes for *mad*, c-*myc* and $\beta_2$–*microglobulin* and sections are labelled accordingly.


Hours following
treatment with OAG
0hr
2hr
24hr
48hr
72hr
mad
c-myc
β2m

## CHAPTER 7: Analysis of E-box DNA-binding during the induced differentiation of HL60 cells.

### 7.1 *Loss of Myc:Max DNA binding is an early event during induced differentiation of HL60 cells.*

Although the data presented in Chapter 6 indicate that *mad* up-regulation is not an essential event for differentiation of HL60 cells (Figures 15 & 18), it must be remembered that uninduced cells express a detectable basal level of *mad* mRNA. This level may be sufficient for translation of effective levels of Mad protein and therefore the results presented in Chapter 6 cannot discount a role for Mad in any of the processes that occur following treatment with the differentiation inducers. As a more direct assay of the possible significance of Mad activity within this system, and with particular interest being given to its relevance to Myc and Myc's target genes, a study was made of the E-box DNA-binding activity of nuclear proteins isolated from the cells at various points during the differentiation programmes.

Analysis of nuclear extracts from cells differentiated with TPA and DMSO (Figure 28 & 29) revealed that a large number of proteins were bound to the E-box sequence at all of the time points sampled. However, this was not unexpected as a number of non Myc- or Mad-related proteins have also been shown to bind this motif (Beckmann et al. 1990; Gregor et al. 1990; Hu et al. 1990). When comparison was made between the profile of DNA-binding of the complexes in Figure 28 with the changes in mRNA levels that had been observed during differentiation of the cells with either TPA or DMSO (Figures 11 & 15), it was apparent that one of the complexes (complex I) had a very similar pattern of binding to the changes in the levels of c-*myc* mRNA that occurs during these processes. Subsequent analysis of complex I with affinity-purified antisera raised against Myc

Figure 28

Figure 28: *EMSA analysis of E-box DNA-binding during differentiation of HL60 cells following treatment with TPA.*

EMSA analysis of E-box DNA binding activity of nuclear proteins isolated from HL60 cells following treatment with TPA. Nuclear protein samples were isolated at the times indicated and equal quantities (10μg) were added to binding reactions containing a $^{32}$P-labelled CM1 double-stranded oligonucleotide, which contains the Myc:Max-binding CACGTG motif (see materials and methods). A complex which has been shown to contain Myc and Max is indicated as I.

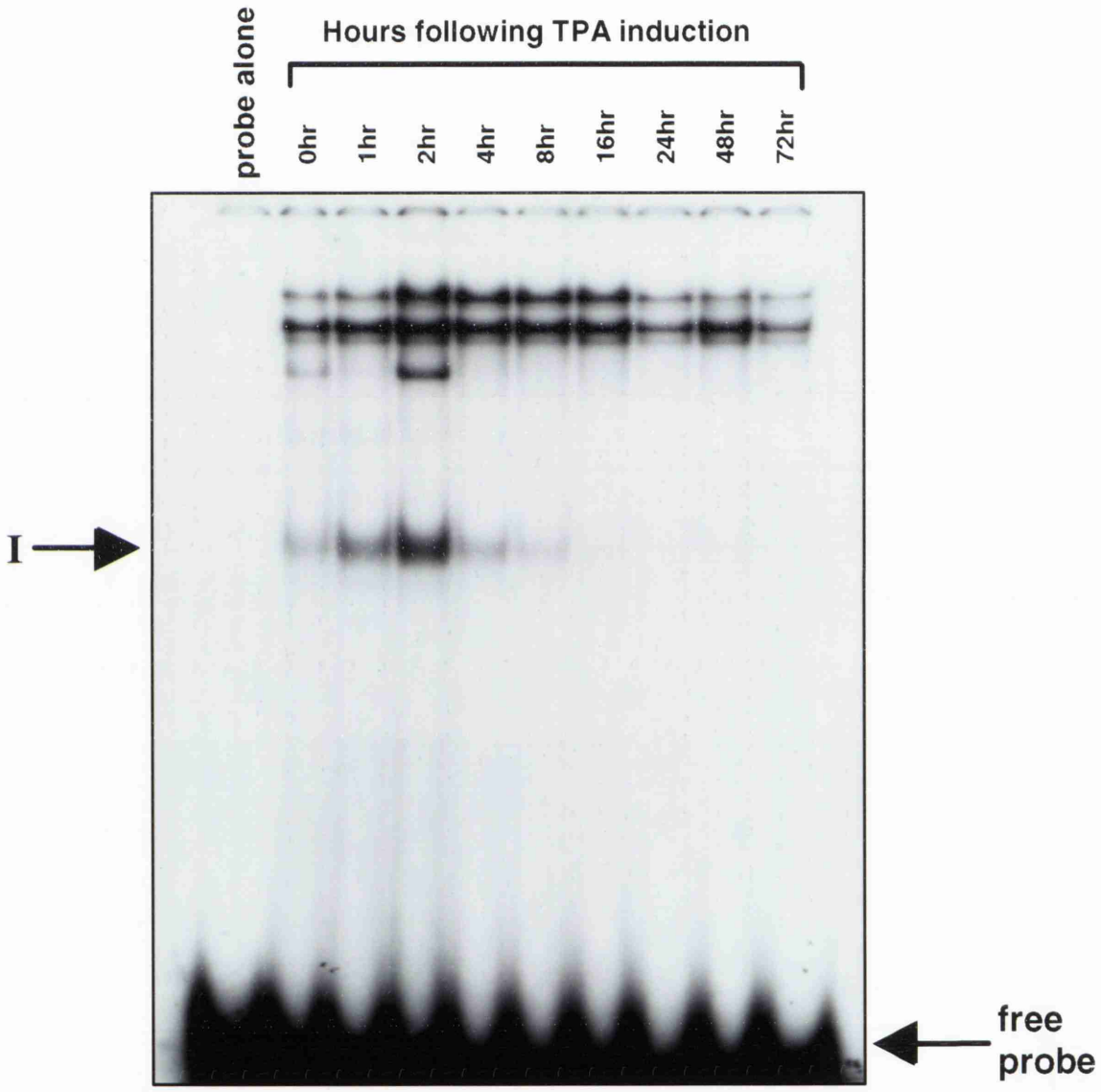

probe alone
Hours following TPA induction
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
I
free
probe

Figure 29

Figure 29: *EMSA analysis of E-box DNA-binding during differentiation of HL60 cells following treatment with DMSO.*

EMSA analysis of E-box DNA binding activity of nuclear proteins isolated from HL60 cells following treatment with DMSO. Nuclear protein samples were isolated at the times indicated and equal quantities (10μg) were added to binding reactions containing a $^{32}P$ labelled CM1 double-stranded oligonucleotide, which contains the Myc:Max-binding CACGTG motif (see materials and methods). A complex which has been shown to contain Myc and Max is indicated as I. Complexes which have been shown to contain Mad (Figure 32) are indicated by II and IIa.

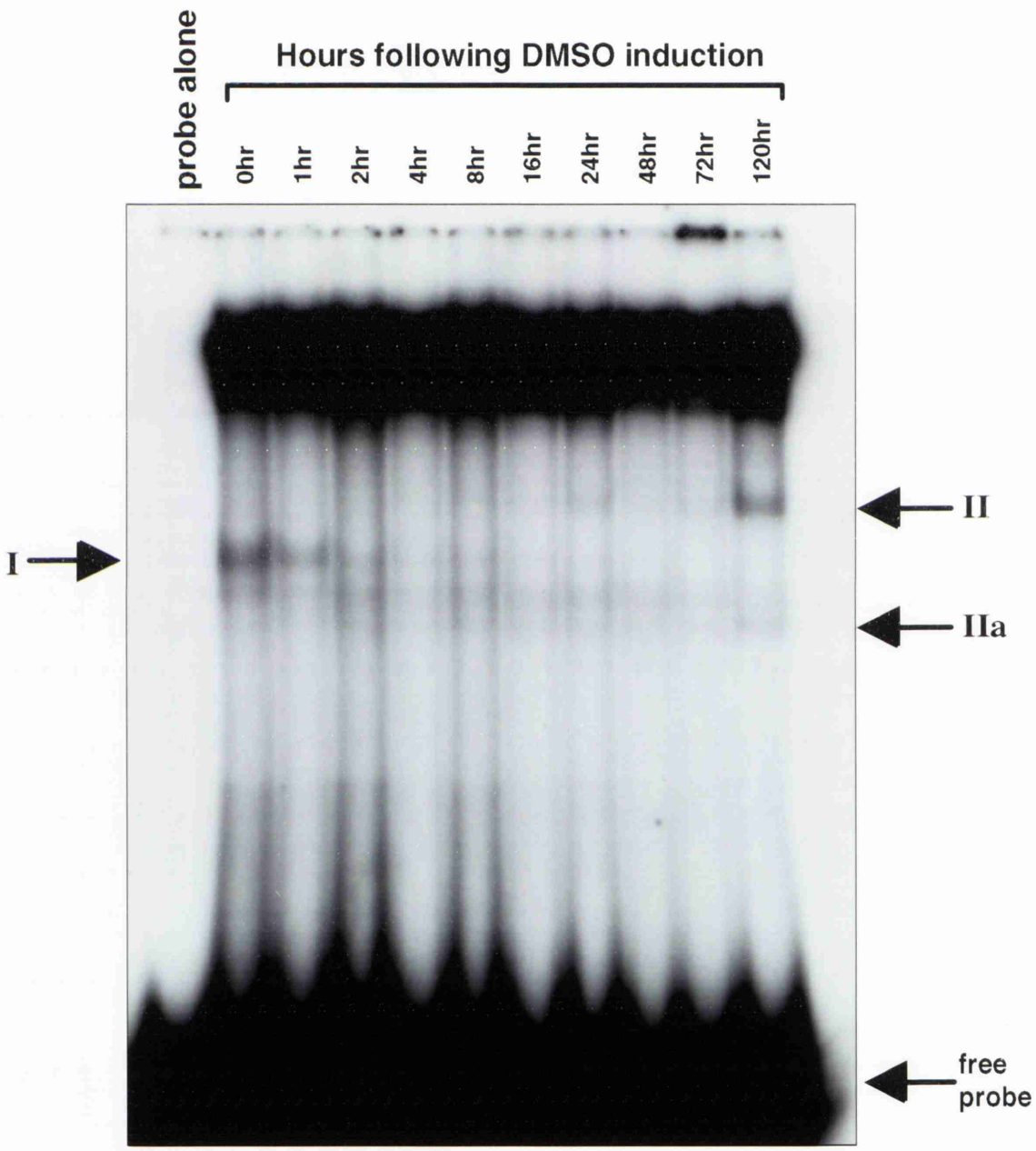
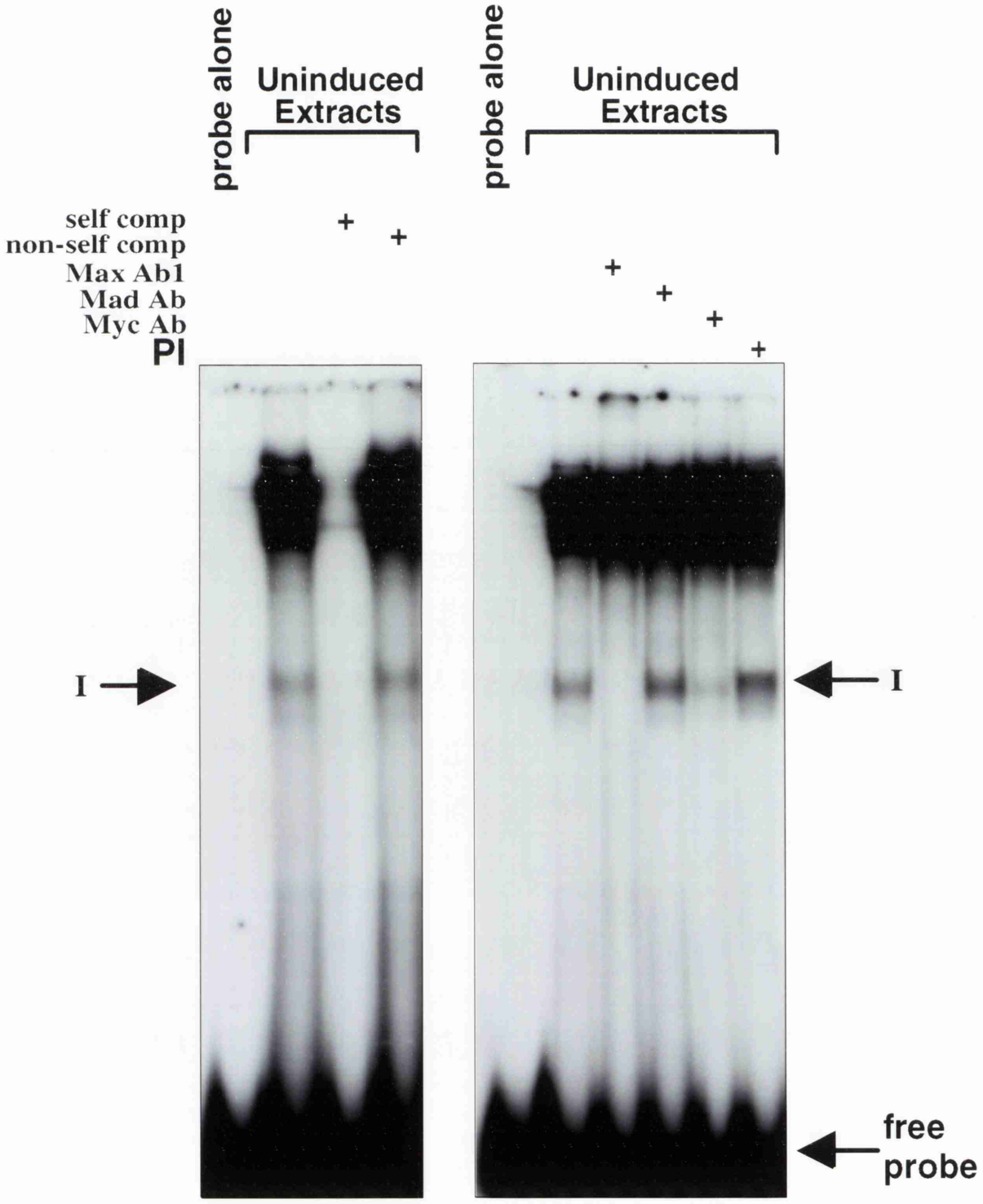

probe alone
Hours following DMSO induction
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
120hr
I
II
IIa
free
probe

Figure 30

Figure 30: *EMSA analysis of the nature of the Myc:Max-containing complex I.*

EMSA analysis to investigate the nature of the complex I that is lost during induced differentiation of HL60 cells by TPA (Figure 28). Equal quantities of nuclear proteins (10μg) from uninduced HL60 cells were incubated prior to addition of CM1 probe with, where shown, either 100-fold excess of unlabelled double-stranded oligonucleotides or antibodies raised against the proteins indicated (see materials and methods). PI, pre-immune serum.


probe alone
Uninduced Extracts
probe alone
Uninduced Extracts
self comp
non-self comp
Max Ab1
Mad Ab
Myc Ab
PI
+
+
+
+
+
+
I
I
free probe

and Max confirmed that this complex was indeed Myc:Max (Figure 30). These data therefore show that loss of Myc:Max DNA-binding is an early event during differentiation of HL60 cells to both granulocytes and monocytes/macrophages. In addition, they provide an example of how changes in c-*myc* mRNA abundance can be rapidly translated into Myc:Max DNA-binding. It is therefore no surprise that regulation of the expression of c-*myc* has been shown to be subject to such intricate control (Marcu et al. 1992).

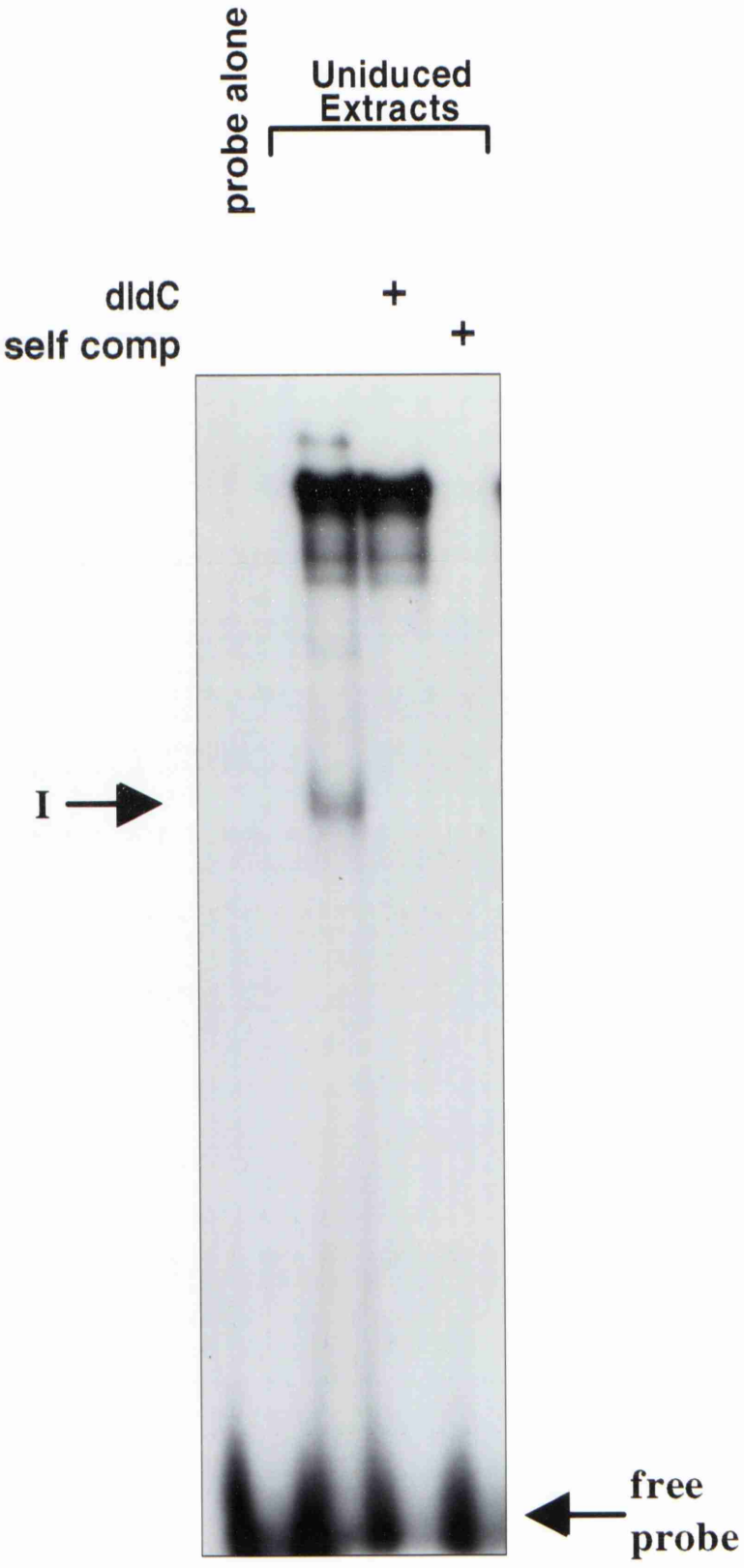
### 7.2 *The Myc:Max-containing complex I is competed away by the double-stranded polynucleotide, poly [dI-dC].*

During the course of this work, it was found that the ability to detect the Myc:Max complexes in cellular extracts was highly dependent on the binding conditions used. In particular, it became apparent that the c-Myc:Max complex could be completely competed by the double-stranded polynucleotide, poly[dI-dC]•poly[dI-dC] (Figure 31). As this factor is a standard constituent of many EMSA protocols, this may be the reason why many workers have been unable effectively to detect Myc:Max DNA binding in nascent cellular extracts. Since it was found that a number of other non-specific double-stranded DNA oligonucleotides did not affect the Myc:Max complex (see Methods 5.7.2), while still retaining the capacity to compete away non-specific proteins from radio-labelled probe, these findings should be a consideration of workers who are planning to conduct studies of a similar nature.

Figure 31

Figure 31: *Demonstration of the effects of the double-stranded polynucleotide poly[dI-dC] on the DNA-binding activity of the Myc:Max complex.*

Nuclear protein samples were isolated from untreated HL60 cells and equal quantities (10μg) added to binding reactions containing a $^{32}P$ labelled 'CM1' double-stranded oligonucleotide which contains the Myc:Max-binding CACGTG motif (see materials and methods). Binding reactions were incubated where shown with either 100 fold excess of unlabelled CM1 or 0.15μg/μl poly[dI-dC]. A complex which has been shown to contain Myc and Max is indicated as I.


probe alone
Uniduced Extracts
dIdC
self comp
+
+
I
free probe

7.3 *Mad-containing DNA-binding complexes are detectable in the late stages of differentiation to granulocytes, but are not seen during differentiation to monocytes/macrophages.*

During the analysis of the DNA-binding complexes that were detectable following treatment of the cells with TPA, no complexes were detected that reacted with the antiserum raised against Mad. However, during granulocytic differentiation of the cells induced by DMSO, two additional complexes were observed (Figure 29) which were not apparent during differentiation to monocytes/macrophages (Figure 28). These two complexes, designated II and IIa (Figure 29), had mobilities slower and faster respectively than that observed for previously described complex I (Figures 28 & 29) and were, particularly in the case of II, induced subsequent to the disappearance of this complex(I). The ensuing analysis of complexes II and IIa using affinity purified antisera (Figure 32) indicated that these complexes did indeed contain Mad. Therefore, Mad-containing complexes are induced during the late stages of differentiation of HL60 cells to granulocytes, but are not induced to detectable levels during differentiation of the cells to monocytes/macrophages. In addition, as these profiles of DNA-binding do not mirror the observed changes in *mad* mRNA during the differentiation of these cells (Figure 15), this finding indicates that Mad DNA-binding is not solely determined by the levels of its mRNA.

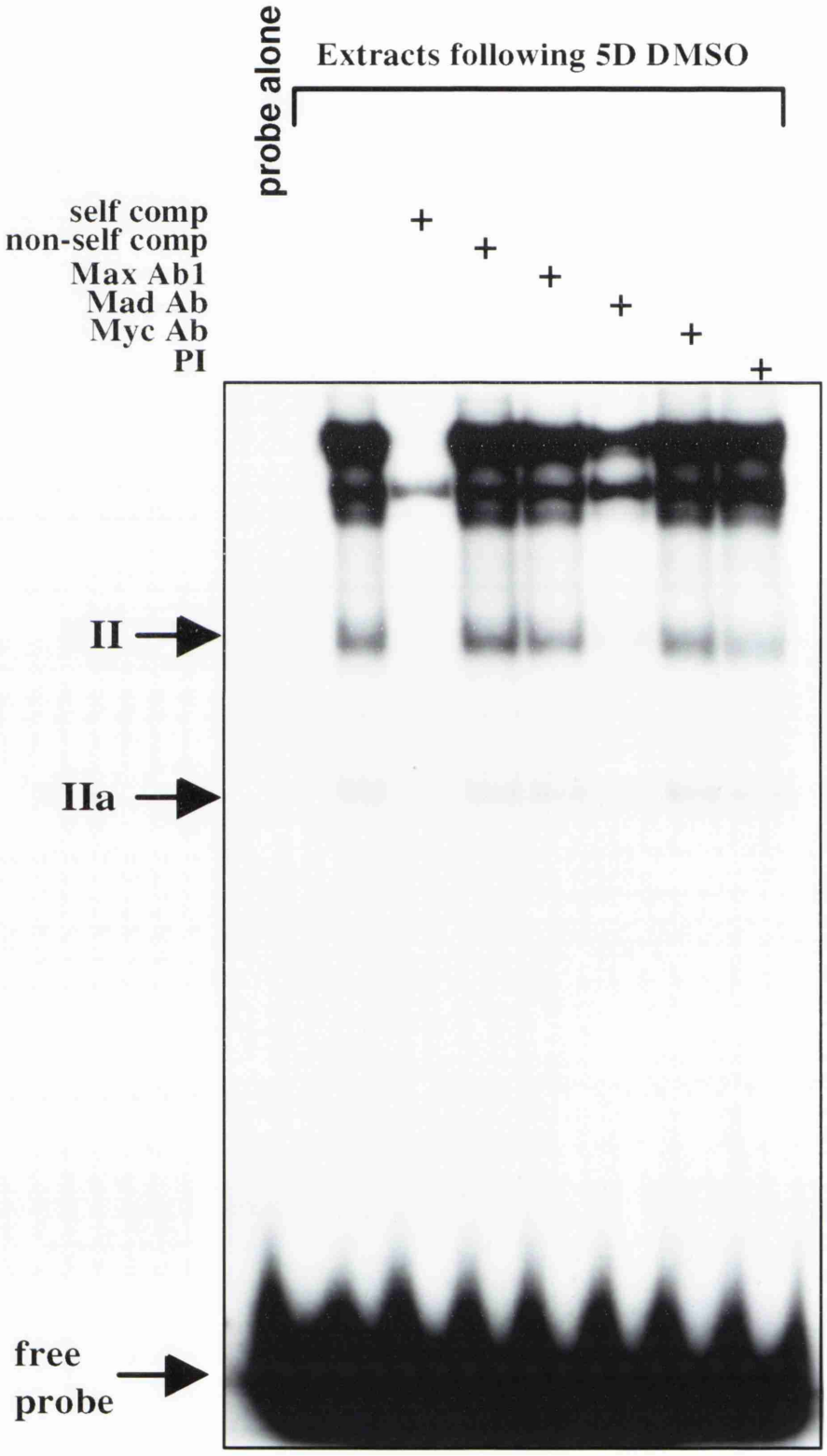
7.4 *Mad-containing DNA-binding complexes induced during granulocytic differentiation do not contain Max.*

Further analysis of complexes II and IIa revealed that they reacted with the Mad antiserum, but not with the antiserum raised against Max (Figure 32). These data are in disagreement with previous results which showed that Mad

Figure 32

Figure 32: *EMSA analysis of the nature of the Mad-containing complexes II and IIa.*

EMSA analysis to investigate the nature of the complexes II and IIa that are induced during differentiation of HL60 cells (Figure 21). Equal quantities of nuclear proteins (10μg) from HL60 cells treated for 5 days with DMSO were incubated prior to addition of CM1 probe with, where shown, either 100-fold excess of unlabelled double-stranded oligonucleotides or antibodies raised against the proteins indicated (see materials and methods). PI, pre-immune serum.


probe alone
Extracts following 5D DMSO
self comp
non-self comp
Max Ab1
Mad Ab
Myc Ab
PI
+
+
+
+
+
+
II
IIa
free
probe

DNA-binding was dependent on dimerization with Max (Ayer et al. 1993). To confirm these results, other Max antisera were used which had been raised against different portions of the human Max protein (see Methods 5.7.2). As can be seen in Figure 33, while each of these antisera was equally effective in recognising Max in a Myc:Max complex (complex I), they did not react with the Mad containing complexes II and IIa. Therefore these complexes do indeed contain Mad, but not Max. Furthermore, in light of the fact that two complexes were detected and that it has been shown that Mad is by itself ineffective in DNA-binding (Ayer et al. 1993), it must also be the case that complexes II and IIa contain factors, in addition to Mad, whose identities are yet to be determined.

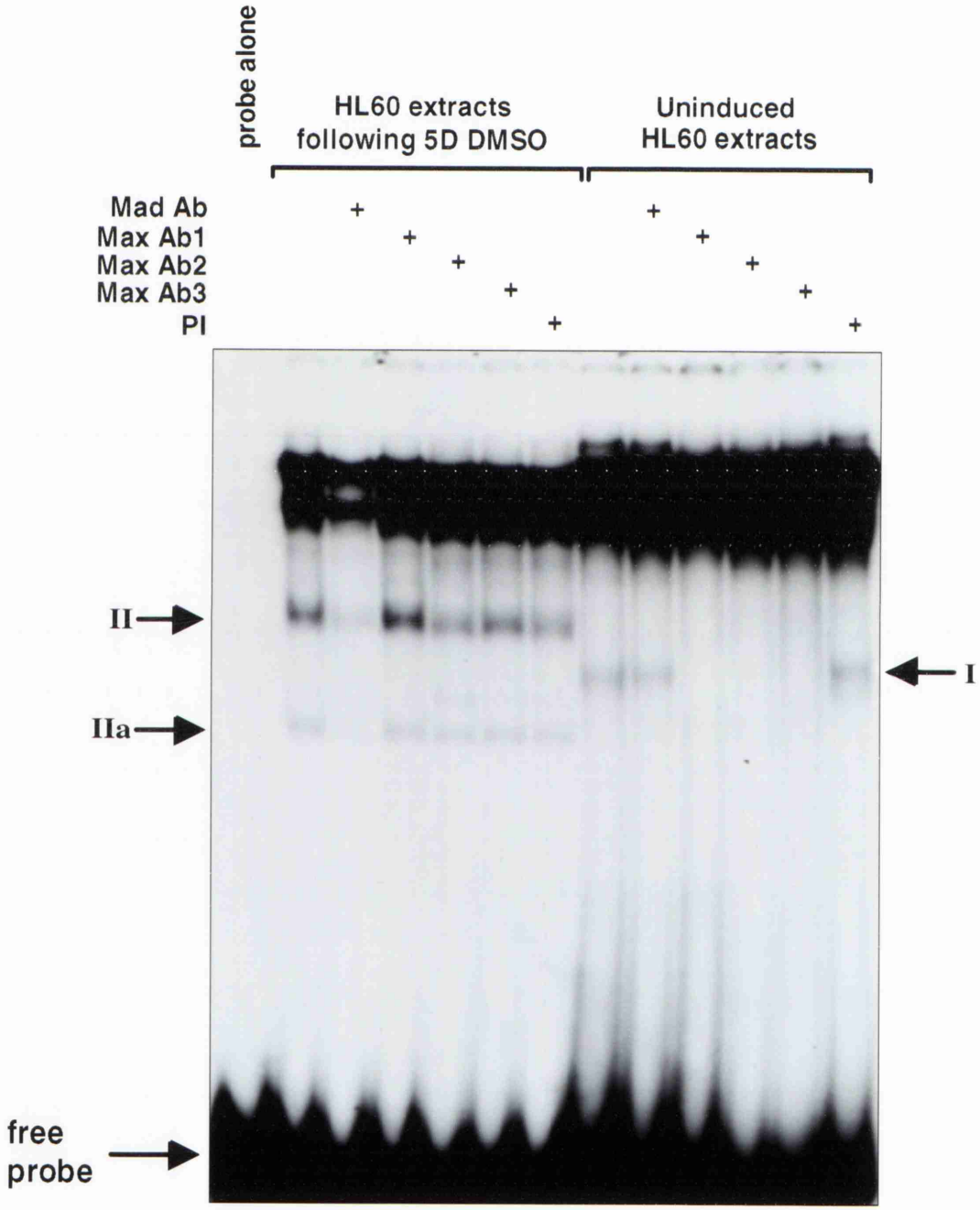
## 7.5 *Summary.*

From the analysis of *max* and *mxi1* mRNAs during differentiation of HL60 and U937 cells, it was unlikely from the patterns of change observed that the proteins translated from these messages were likely to be determining factors in the control of differentiation in these systems. In contrast, it was a much more provocative observation that the levels of *mad* mRNA were increased prior to the loss of c-Myc protein that occurs during differentiation induced by TPA. This opened up the possibility that the abrogation of the differentiation-blocking activity of c-Myc in these cells, might not primarily occur through the loss of c-Myc protein, but through an increase in the abundance of its antagonist, Mad. However, it was also found that an increase in the levels of *mad* mRNA was not required for differentiation induced by other agents. In addition, as EMSA analysis revealed that E-box DNA-binding by Mad was only detectable in the latter stages of differentiation, it would seem, akin to Max and Mxi1, that Mad is also unlikely to be a determining factor in the release of the differentiation block of these cells. In light of these findings, it was decided not to investigate these factors further, but to focus

Figure 33

Figure 33: *EMSA to confirm that complexes II and IIa contain Mad, but not Max.*

EMSA analysis showing the reactivity of proteins in complexes I, II and IIa to antiserum raised against Mad1 and a series of antisera raised against Max. Nuclear proteins are either from uninduced cells or from cells treated for 5 days with DMSO. Sources of antisera are indicated in materials and methods. PI, pre-immune serum.


probe alone
HL60 extracts following 5D DMSO
Uninduced HL60 extracts
Mad Ab
Max Ab1
Max Ab2
Max Ab3
PI
II
IIa
I
free probe

on the role played by c-*myc* in the control of the differentiation process *per se*. It is therefore the aims of the following chapter to, at least in part, specifically address this question.

## CHAPTER 8: Analysis of the role of c-Myc in the control of myeloid differentiation.

### 8.1 *Analysis of the effects of TPA treatment on the growth status and differentiation capacity of a differentiation defective variant of the U937 cell line.*

In order to gain more insight into the mechanisms by which c-Myc can abrogate the ability of myeloid cells to differentiate, analysis was made of a differentiation-defective variant of the U937 cell line. Analysis of this line, which is described here as U937d-, revealed that it differed from its "wild-type" counterpart (U937d+), in that it did not undergo a programme of differentiation to monocytes/macrophages following treatment with the phorbol ester, TPA. It is obvious from morphological analysis that TPA treatment of this line does not result in the formation of characteristic aggregates which adhere to the plastic of the tissue culture dish but, in fact, it leaves the cells looking exactly like cultures that have not been treated with the differentiation inducer (Figure 34). Moreover, when analysed histochemically it is clear that U937d- cells are again different from U937d+ cells, in that treatment with TPA causes only a small proportion of the cells to have high levels of activity of the terminal differentiation marker, non-specific esterase (NSE) (Figure 35). However, in spite of these findings, it was found that U937d- did in fact respond to the differentiation inducer by undergoing a growth arrest which occurred in a similar manner, if not even more quickly, than that seen in U937d+ (Figure 36).

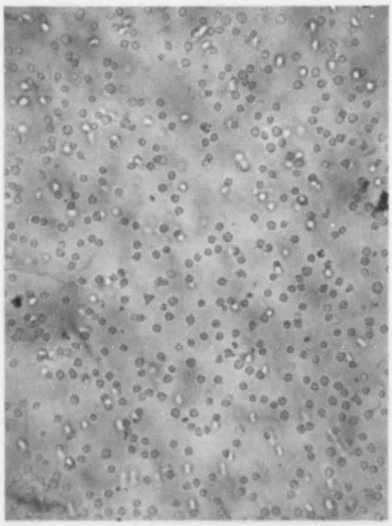
Indeed, there are many similarities between U937d- cells and the previously described HL60 variant, Ast4 (see section 6.9). However, unlike HL60-Ast4, U937d- cells are more amenable for analysis as they do not

Figure 34

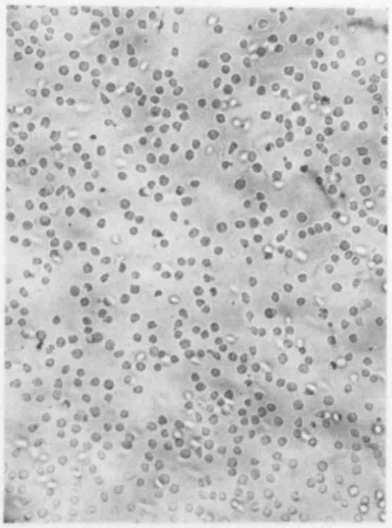
Figure 34: *Comparison of the differentiation capacity of U937d+ and U937d- cells following treatment with TPA.*

Morphological appearance of U937d+ and U937d- cells following treatment for 5 days with TPA at a final concentration of $1.6 \times 10^{-7}$M. Uninduced cells were incubated for the same period of time with an equal amount of solvent that was used in the phorbol ester treatments. These photographs are representative of what was observed on at least five separate occasions.

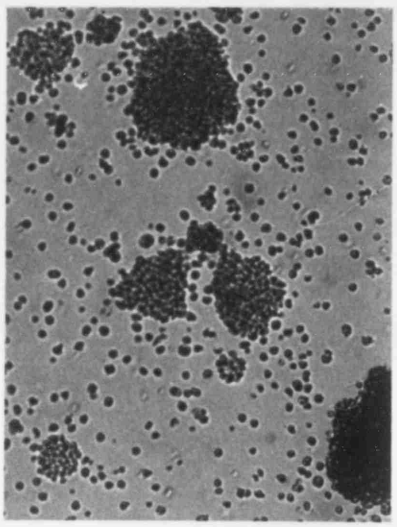
Uninduced
U937d+



Uninduced
U937d-



U937d+
+TPA



U937d-
+TPA

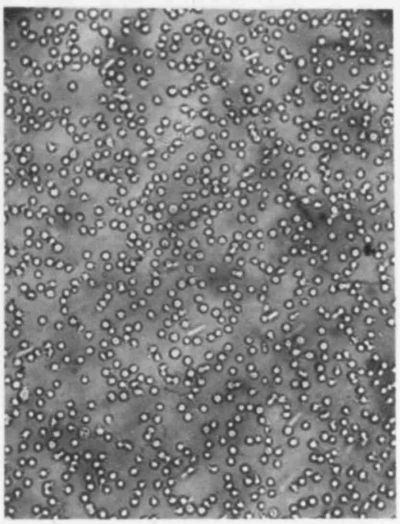
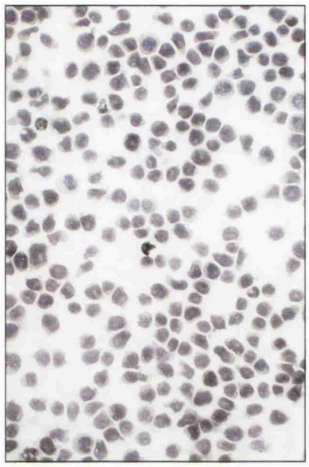


Figure 35

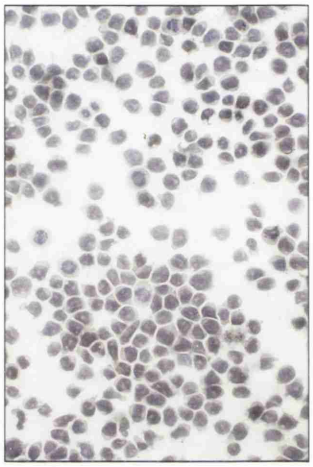
Figure 35: *Analysis of the activity of the terminal differentiation marker non-specific esterase, following TPA treatment of U937d+ and U937d- cells.*

U937d+ and U937d- cells were treated with TPA at a final concentration of 1.6 x 10-7M for a period of 5 days. They were then stained for NSE activity as described in materials and methods. Sections of the figure are labelled accordingly.

Untreated
U937d+



Untreated
U937d-



U937d+
+ 120hr TPA

U937d-
+120hr TPA

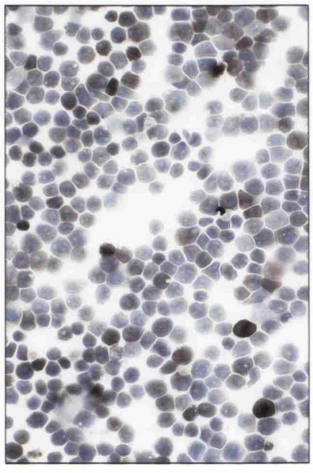


Figure 36

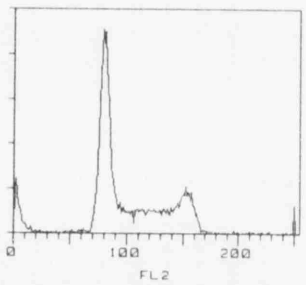
Figure 36: *Analysis of the effects of TPA-treatment on the cell-cycle status of U937d+ and U937d- cells.*

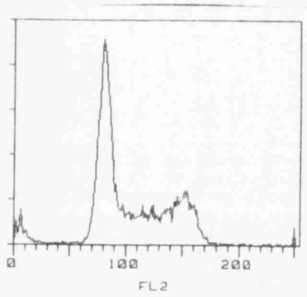
Flow cytometry profiles of the DNA content of populations of U937d+ and U937d- following treatment with TPA at a final concentration of 1.6 x $10^{-7}$M. The x axis of the profiles represents the fluorescence activity that is proportional to the DNA content of cells within the sample analysed. The y axis represents the number of cells. Equal numbers of events (10,000) were analysed for the two cell lines at each of the time points indicated. The peak in the extreme left of each profile represents chicken reticulocytes which were added to each sample as a reference point. These profiles are indicative of what was observed in three separate experiments.

U937d+

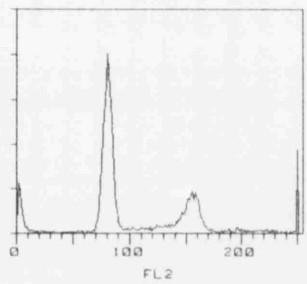
U937d-

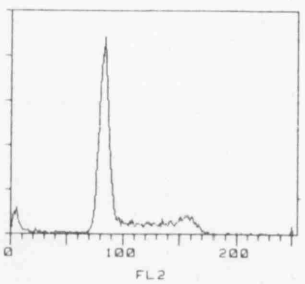
Uninduced



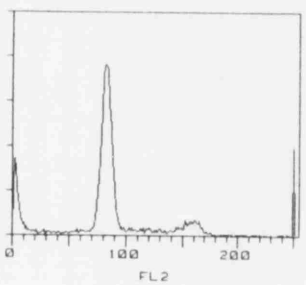



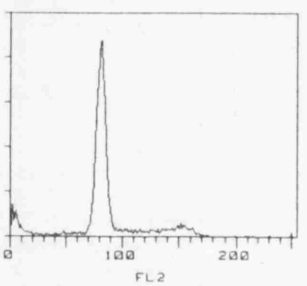

24hr TPA



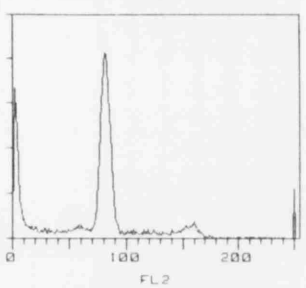



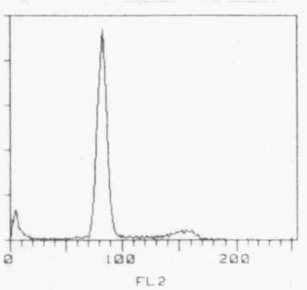

48hr TPA



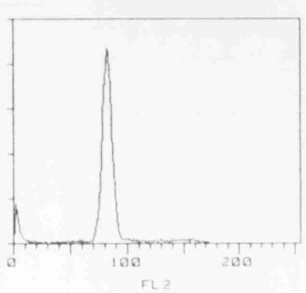



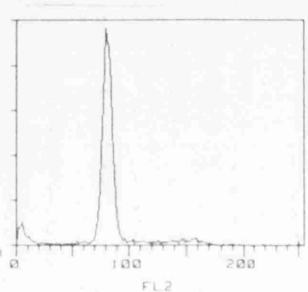

72hr TPA




120hr TPA



subsequently die after the formation of a TPA-induced $G_1$ arrest. In fact, it was found on a number of occasions that the growth arrest of these cells could be maintained for up to a period of three weeks without any obvious signs of cell death and without any decrease in cell numbers.

## 8.2 *Loss of Myc protein expression is not obligatory for growth arrest in myeloid leukaemic cells.*

As down-regulation of the levels of c-Myc protein has been shown to be a characteristic change that accompanies the induced differentiation of "wild-type" U937 cells (Eickholt et al. 1993), it was decided to analyse the levels c-*myc* mRNA and protein in U937d-. In a similar manner to what was observed in HL60-Ast4 cells (Figure 22), but in contrast to the situation in U937d+ (Figure 17), it was found that, the levels of c-*myc* mRNA were only transiently decreased following treatment with TPA (Figure 37). Moreover, after the re-establishment of pre-induction levels of c-*myc* mRNA after 72hr of treatment, it was found that these levels were then maintained for the rest of the period analysed (168hr). The subsequent analysis of c-Myc protein in these cells, revealed that while the abundance of the protein was decreased in U937d+ cells, the levels of c-Myc in U937d- cells were not decreased and were for the most part maintained at the levels observed in untreated cells (Figure 38). This finding, in conjunction with the fact that these cells are growth arrested (Figure 36), therefore indicates that a decrease in the levels of c-*myc* mRNA and protein are events not required for the effective withdraw from the cell cycle.

Figure 37

Figure 37: *Northern blot analysis of the levels of c-myc mRNA during TPA treatment of U937d+ and U937d- cells.*

Northern blot analysis of RNAs isolated, at the times indicated, from U937d+ and U937d- cells following treatment with TPA at a final concentration of 1.6 x $10^{-7}$M. The blots was initially hybridised with a cDNA probe for c-*myc*, subsequently stripped and then as an assessment of loading were re-hybridised with a probe for $\beta_2$–*microglobulin.* Sections of the figure relating to each probing are labelled accordingly.

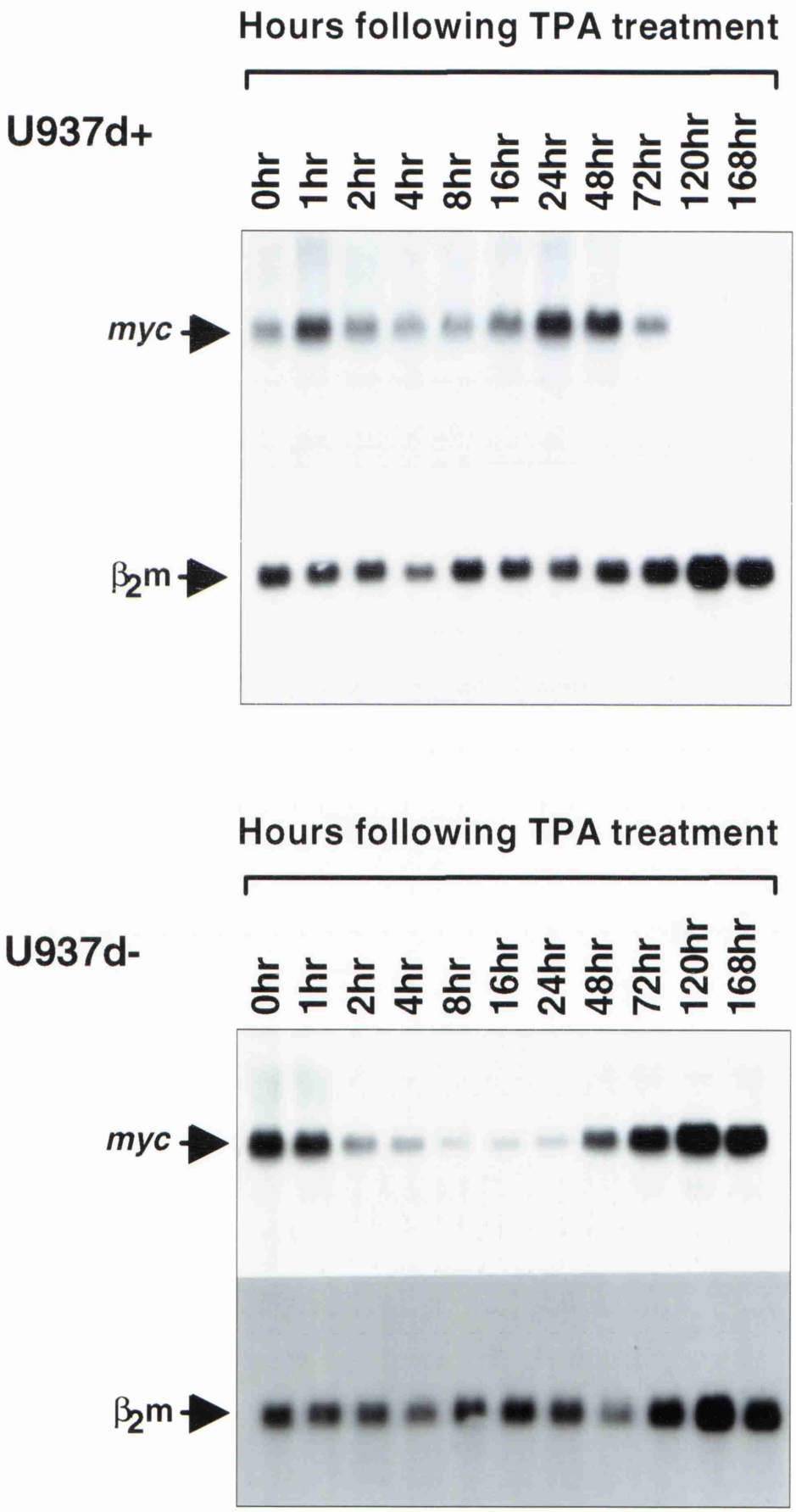
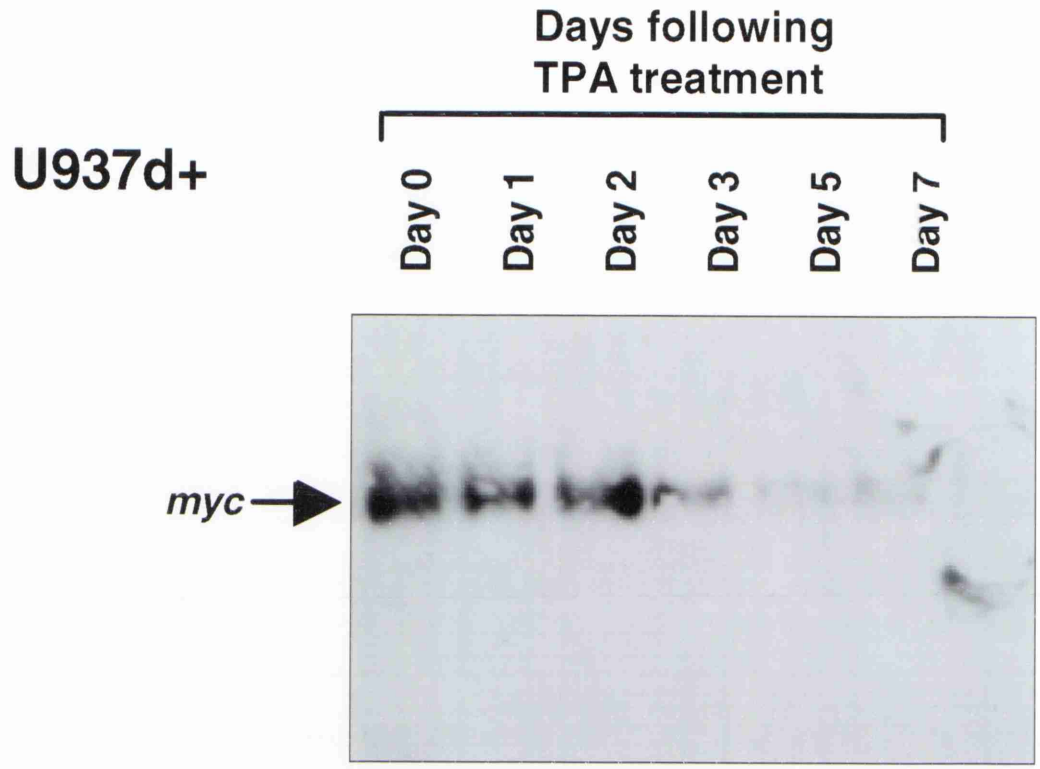
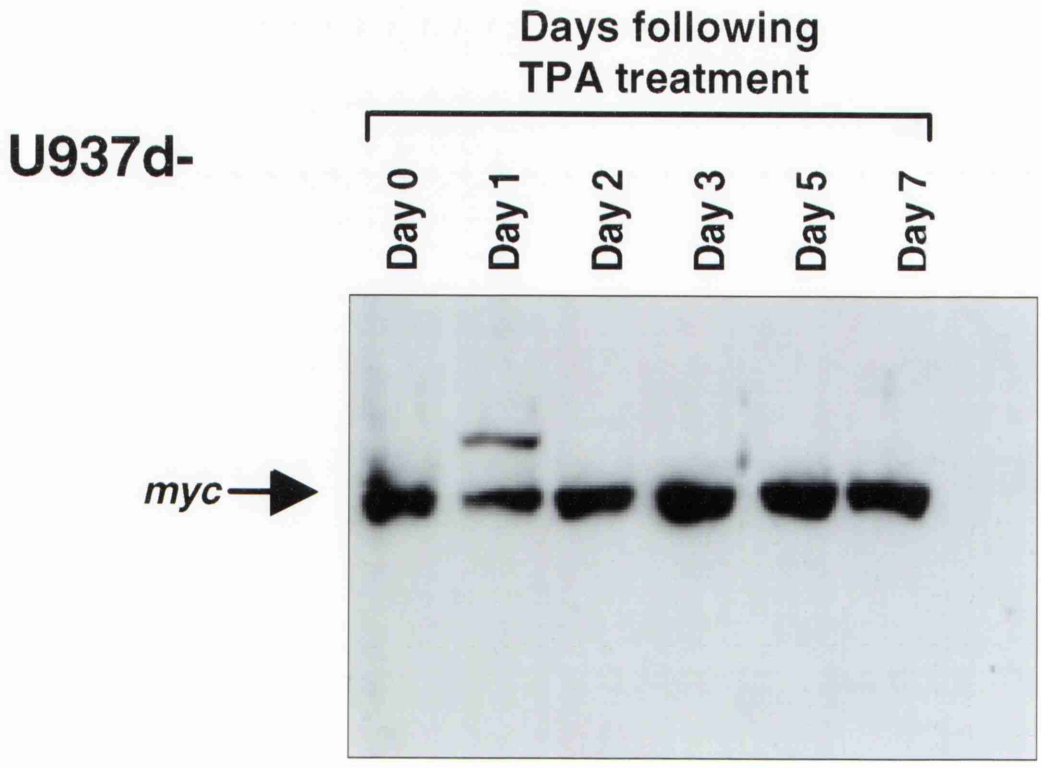

Hours following TPA treatment
U937d+
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
120hr
168hr
myc
β2m
Hours following TPA treatment
U937d-
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
120hr
168hr
myc
β2m

Figure 38

Figure 38: *Western blot analysis of the levels of c-Myc protein during TPA treatment of U937d+ and U937d- cells.*

Western blot analysis of the levels of c-Myc protein in U937d+ and U937d- cells following treatment with TPA, for the times indicated, at a final concentration of $1 \times 10^{-7}$ M. Equal quantities of protein from each time point were resolved through polyacrylamide gels, blotted and assessed for Myc protein using an antiserum which had been raised against the full-length human c-Myc protein. The more slowly migrating second band that is seen in U937d- proteins following treatment with TPA for 1 day was not consistently seen during these studies and we therefore feel is artefactual. However, the profiles of c-Myc protein were consistently reproducible.


U937d+
Days following
TPA treatment
Day 0
Day 1
Day 2
Day 3
Day 5
Day 7
myc


U937d-
Days following
TPA treatment
Day 0
Day 1
Day 2
Day 3
Day 5
Day 7
myc

8.3 *Myc protein from TPA-treated U937d- cells retains the capacity to bind DNA.*

Since the above findings are in conflict with previous studies which have indicated that the expression of c-Myc alone is sufficient to direct cell-cycle progression (Eilers et al. 1991), it was decided to examine further TPA-treated U937d- cells in order to ascertain whether the Myc protein in these cells could be considered to have functional capacity. As it has previously been shown that effective DNA-binding and transcriptional activation by c-Myc is dependent on it forming a complex with Max (Blackwood and Eisenman 1991; Amati et al. 1992), analysis was first made of the expression of *max* mRNA in U937d- following treatment with TPA. In agreement with reports by a number of other groups, which have shown that the expression of *max* is largely invariant during the induced differentiation of U937 cells (Ayer and Eisenman 1993; Larsson et al. 1994), it was found that the levels of *max* mRNA were unaffected by the TPA treatment (Figure 39). Therefore, this indicates that the c-Myc protein in U937d- cells should be able to bind DNA at the E-box binding motif, CACGTG.

As a result, analysis was subsequently made of nuclear protein extracts from TPA-treated and untreated U937d- cells for their ability to bind the previously described consensus Myc:Max binding sequence, CM1 (Blackwood and Eisenman 1991; see section 5.7.2). As can be seen in Figure 40, a large number of protein complexes were found to bind the CM1 oligonucleotide in both treated and untreated cells. However, as described previously in Section 7.1, this was not unexpected as many non Myc-related ubiquitously expressed proteins have also been shown to bind this motif (Beckmann et al. 1990; Gregor et al. 1990; Hu et al. 1990). Nevertheless, only one of the complexes detected in this assay was found to be reactive

Figure 39

Figure 39: *Northern blot analysis of the levels of max mRNA during TPA treatment of U937d+ and U937d- cells.*

*Max* expression in U937d- cells is unaffected by treatment with TPA. Northern blot analysis of RNAs isolated, at the times indicated, from U937d- cells following treatment with TPA(A) at a final concentration of $1.6 \times 10^{-7}$M. The blots was initially hybridised with a cDNA probe for *max*, subsequently stripped and then as an assessment of loading were re-hybridised with a probe for $\beta_2$*–microglobulin.* Sections of the figure relating to each probing are labelled accordingly.

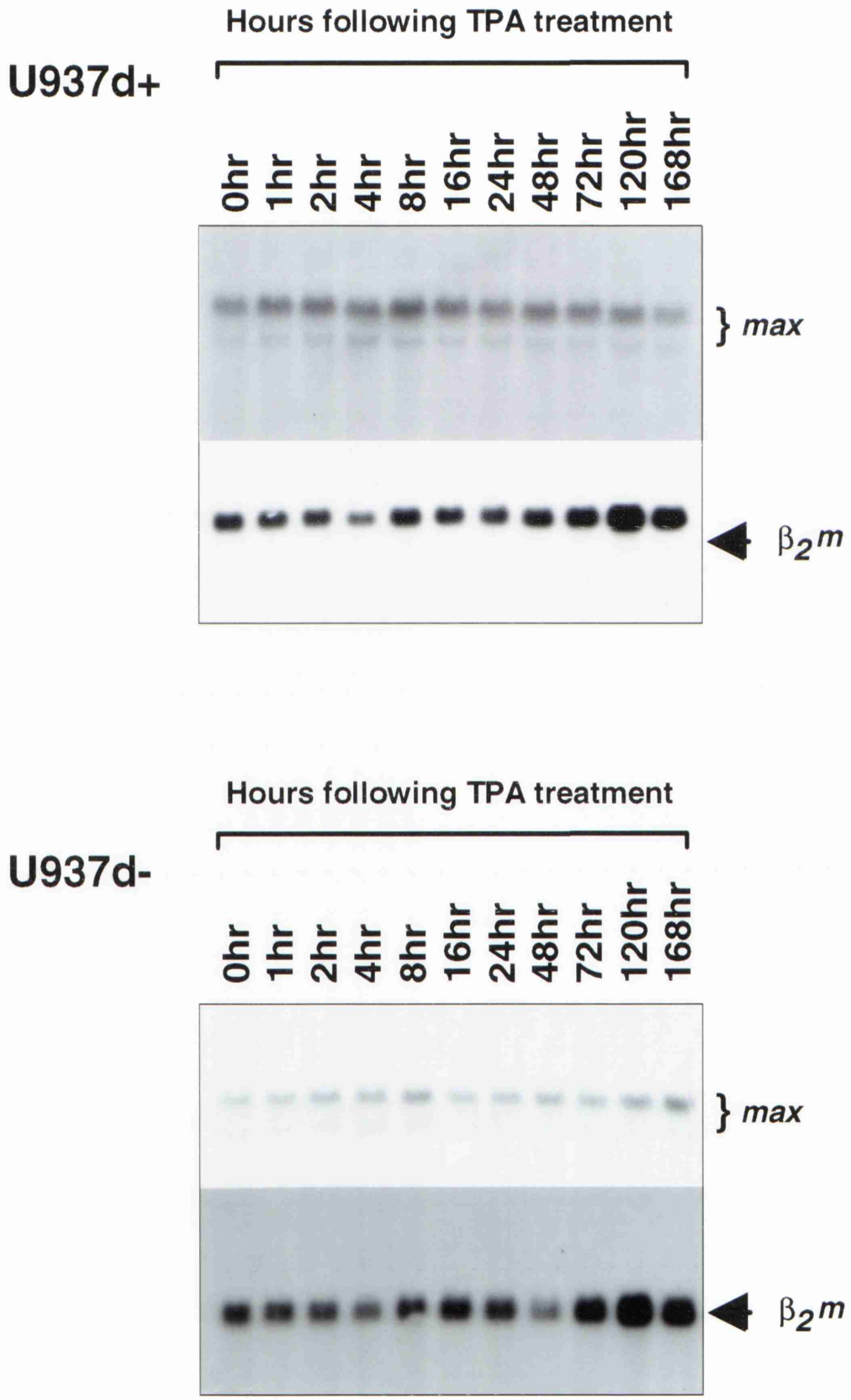
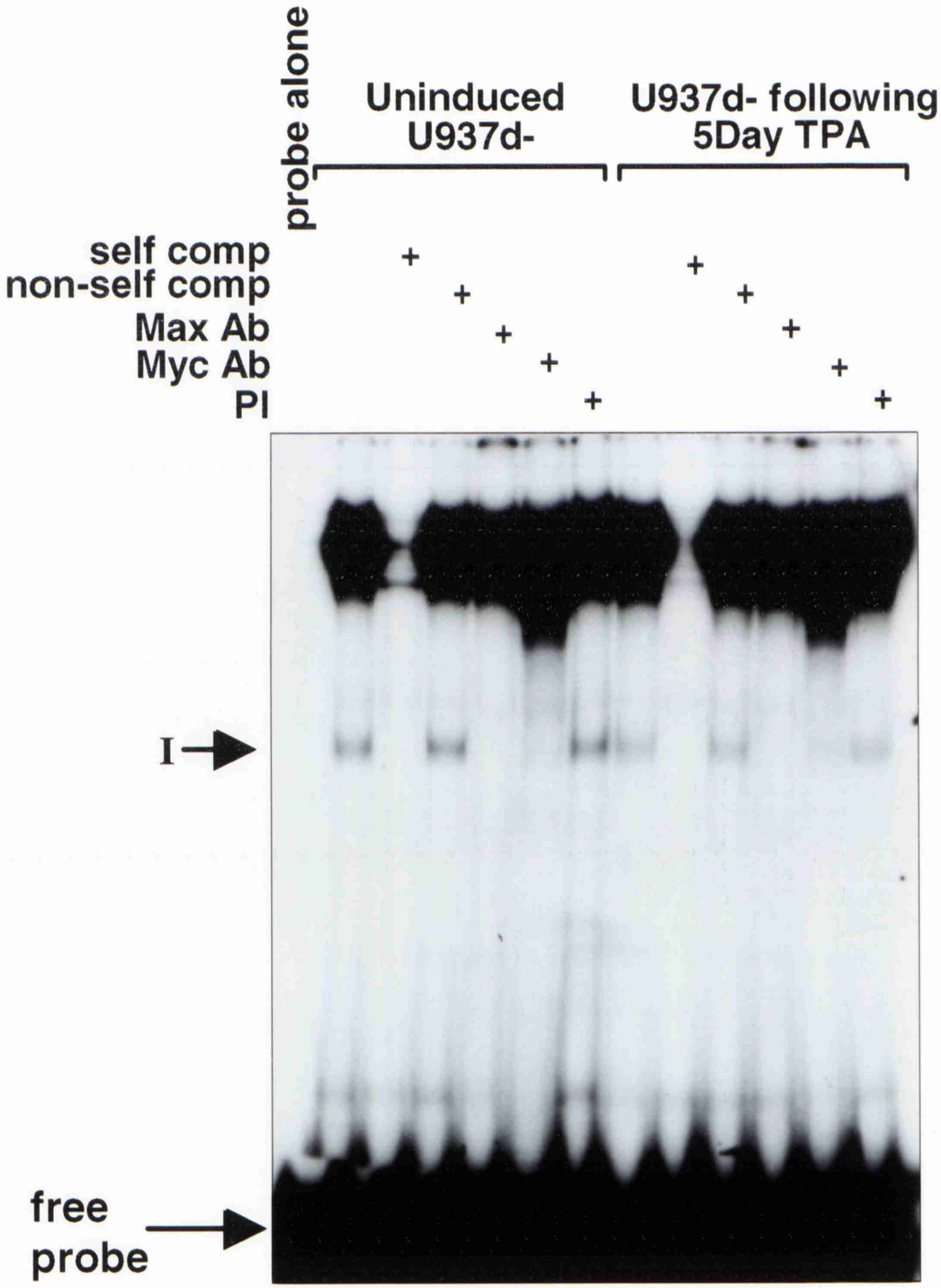

Hours following TPA treatment
U937d+
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
120hr
168hr
} max
β2m
Hours following TPA treatment
U937d-
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
120hr
168hr
} max
β2m

Figure 40

Figure 40: *EMSA analysis to investigate the effects of TPA treatment on Myc:Max DNA binding in protein extracts from U937d- cells.*

EMSA analysis of E-box DNA binding activity of nuclear proteins isolated from U937d- cells which had been incubated with either 1.6 x 10-7M TPA or a corresponding amount of solvent. Nuclear protein samples were isolated at the times indicated and equal quantities (10μg) were added to binding reactions containing a $^{32}P$ labelled CM1 double-stranded oligonucleotide, which contains the Myc:Max-binding CACGTG motif (see materials and methods). The Myc:Max complex, as determined by antibody reactivity, is indicated as I. Specific and non-specific competitors were incubated at a concentration which was 100x greater than that of the labelled probe. PI, pre-immune serum.


probe alone
Uninduced U937d-
U937d- following 5Day TPA
self comp
non-self comp
Max Ab
Myc Ab
PI
I
free probe

with antisera raised against human Myc and Max peptides (complex I). Moreover, this complex migrated at exactly the same rate as the Myc:Max-containing complex which was extensively characterised during HL60 cell differentiation in Chapter 7. Indeed, as it was found that the DNA-binding profile of this complex during HL60 differentiation mirrored exactly the expression patterns of both c-*myc* mRNA and protein during this process, it is perhaps no surprise that a DNA-binding activity containing Myc:Max (complex I) is retained in TPA-treated U937d- cells, which continue to express high levels of c-Myc protein (Figure 38).

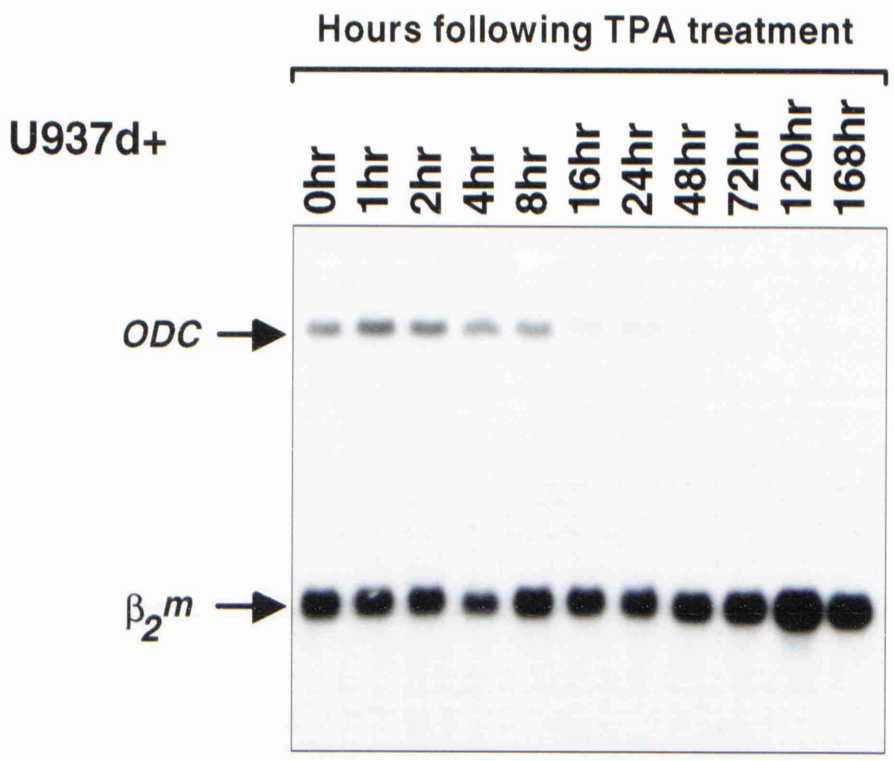
8.4 *Expression of ODC is down-regulated in U937d- cells following treatment with TPA, despite continued c-Myc expression.*

It has been shown that the activity of the protein product of the Myc target gene *ODC* is essential for cell-cycle progression (Pegg 1986). Furthermore, it has also been reported that activation of c-Myc alone, at least in fibroblasts, is sufficient to induce the expression of *ODC* (Wagner et al. 1993). However, while the results presented above show that the c-Myc protein in TPA-treated U937d- cells retains the ability to bind the E-box DNA-binding motif, through which *ODC* is transactivated (Bello-Fernandez et al. 1993), this does not necessarily mean that these cells also maintain expression of *ODC*. In order to address this question, analysis was made of the levels of *ODC* mRNA in both U937d- and U937d+ cells following incubation with TPA for a period of 7 days. As can be seen in Figure 41, treatment with the phorbol ester down-regulated the levels of *ODC* in both U937d+ and U937d- cells. Moreover, it was found that the levels of *ODC* mRNA were decreased more rapidly in U937d- than in U937d+. On the basis of the fact that the protein is extremely unstable ($t_{1/2}$=15min)(Pegg 1986; Hayashi and Murakami 1995), it can be

Figure 41

Figure 41: *Northern blot analysis of the levels of ODC mRNA during TPA treatment of U937d+ and U937d- cells.*

Northern blot analysis of RNAs isolated, at the times indicated, from U937d+ and U937d- cells following treatment with TPA at a final concentration of $1.6 \times 10^{-7}$M. The blots was initially hybridised with a cDNA probe for *ODC*, subsequently stripped and then as an assessment of loading were re-hybridised with a probe for $\beta_2$*–microglobulin.* Sections of the figure relating to each probing are labelled accordingly.


Hours following TPA treatment
U937d+
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
120hr
168hr
ODC
β2m

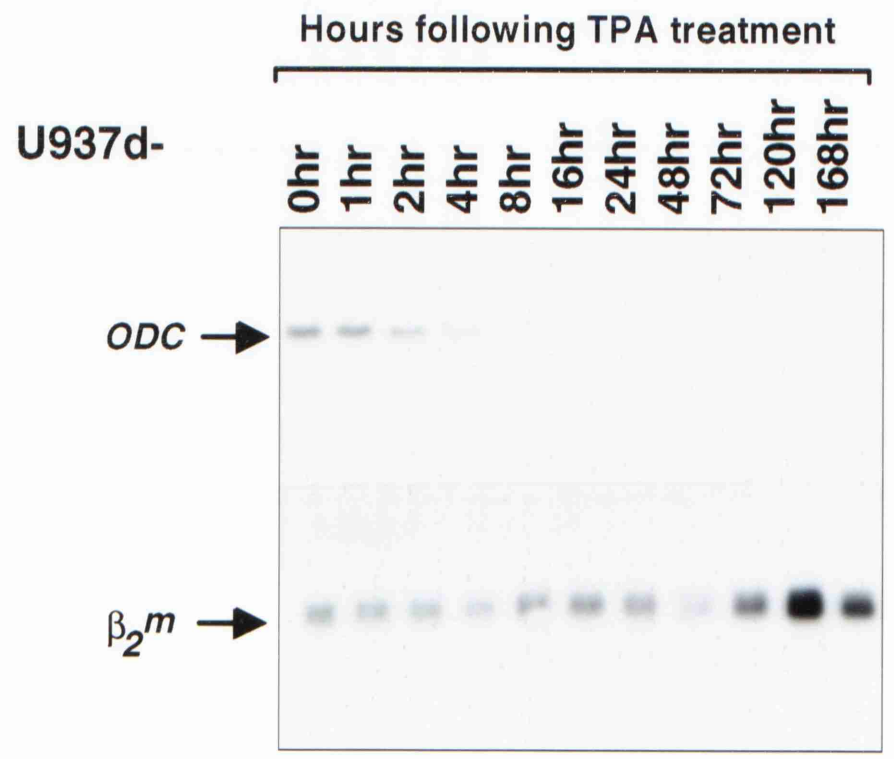
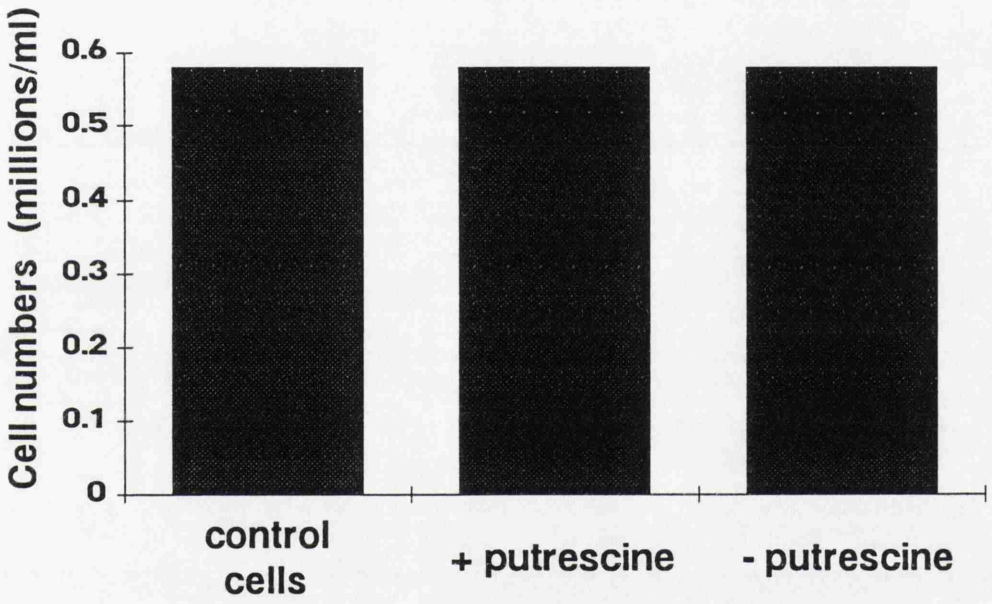

Hours following TPA treatment
U937d-
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
120hr
168hr
ODC
β2m

Figure 42

Figure 42: *Analysis of the significance of the decrease in ODC mRNA levels to the phenotype of U937d- cells following treatment with TPA: Complementation of ODC activity by exogenous putrescine.*

U937d- cells were treated for 5 days with TPA at a final of $1.6 \times 10^{-7}$M. The culture was then counted and split in two separate flasks and incubated either in the absence or presence of 0.1mM putrescine for a further 2 weeks (This concentration has previously been shown to be sufficient to reverse the effects of the ODC inhibitor DFMO in U937 cells (Tahara et al. 1991)). Cell numbers were then counted again. A second independent experiment gave identical results.


Cell numbers (millions/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0
control cells
+ putrescine
- putrescine

predicted that loss of ODC activity will occur soon after the disappearance of its mRNA. With this in mind, these findings provide a possible reason why, in spite of continued c-Myc expression, U937d- cells withdraw from the cell-cycle following treatment with TPA. However, the mechanism by which the level of *ODC* mRNA is down-regulated remains undetermined.

Since it has been shown that the product of the ODC enzyme is the polyamine, putrescine (Pegg 1986), it was attempted to relieve the growth arrest of U937d- cells by adding putrescine to complement for the absence of ODC activity. In this regard, it had previously been shown that addition of exogenous putrescine directly into the growth medium, was sufficient to induce cell-cycle progression in cell lines which contain inactivating mutations in the *ODC* gene (Pohjanpelto et al. 1985). However, when putrescine was added to TPA-treated U937d- cells, even in the presence of fresh medium and serum, no increase in cell numbers was observed (Figure 42). It must therefore be the case that down-regulation of the expression of *ODC* is not the only reason why U937d-, despite continued c-Myc expression, maintain the cell cycle arrest which occurs as a consequence of treating the cells with TPA.

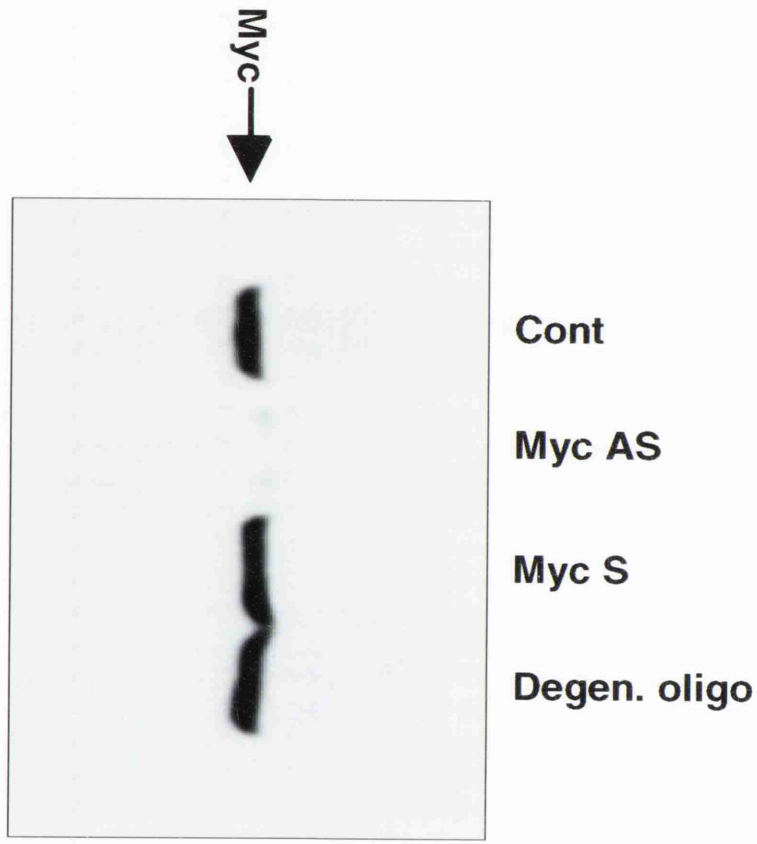
## 8.5 *The block to differentiation in U937d- cells can be released by antisense oligonucleotides directed against c-myc mRNA.*

The data presented here show that U937d- cells are unlike their wild-type counterparts in that, following treatment with TPA, they rapidly withdraw from the cell cycle, without a concomitant programme of terminal differentiation (Figures 34, 35 & 36). In addition, these cells are able to undergo this growth arrest without an accompanying decrease in the levels of c-*myc* mRNA and protein (Figures 37 & 38). Therefore, on the basis of these findings it was hypothesised that the block to differentiation of U937d- cells

Figure 43

Figure 43: *Effect of c-myc antisense oligonucleotides on the levels of c-Myc protein in TPA-treated U937d- cells.*

Western blot analysis showing the effects of treating U937d- cells with antisense oligonucleotides directed against the c-*myc* mRNA. In addition to treatment with TPA to a final concentration of $1.6 \times 10^{-7}$ M, the cells were also subjected to daily addition of oligonucleotide at a concentration of 20μM, for a period of 5 days. Control cells (Cont) were treated with TPA alone for the same period of time. Myc AS, antisense oligonucleotide directed against the 5' of c-*myc* mRNA; Myc S, complementary sense oligonucleotide; Degen. oligo, degenerate oligonucleotide. A second independent experiment gave identical results.


Myc
Cont
Myc AS
Myc S
Degen. oligo

may be as a result of the continued expression of c-Myc in these cells and yet, however, dissociated from c-Myc's ability to direct cell-cycle progression. To answer this question, the levels of c-Myc protein were down-regulated in TPA-treated U937d- cells, by using antisense oligonucleotides directed against the c-*myc* mRNA. One oligonucleotide, which was found to be particularly effective, had previously been used to reduce the levels of c-Myc protein, and subsequently induce growth arrest (Wickstrom et al. 1989) and differentiation (Bacon and Wickstrom 1991), of HL60 cells. When U937d- cells were incubated for 5 days with both this oligonucleotide and TPA a marked decrease in the levels of c-Myc protein was observed when compared to that detected in cells treated for the same period with TPA alone (Figure 43). Furthermore, this down-regulation was not observed when the cells were incubated with TPA in conjunction with either a complementary c-*myc* sense oligonucleotide or a non-specific degenerate oligonucleotide (Figure 43).

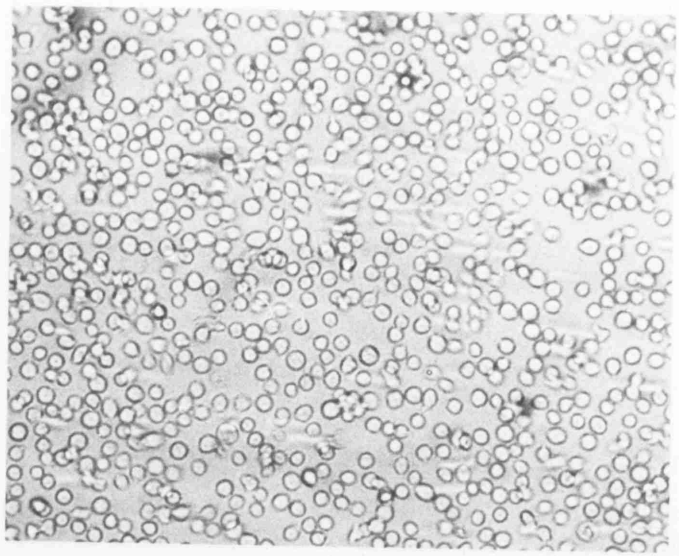
Following treatment with the oligonucleotides, an assessment was also made of the differentiation status of the cells. In agreement with the initial hypothesis, it was found that treatment with c-*myc* antisense oligonucleotide, but not with the complementary sense or degenerate oligonucleotide, resulted in morphological changes in the cells (Figure 44) which were characteristic of the appearance of U937d+ cells following their differentiation induced by TPA (Figure 34). To assess whether these morphological changes resulted from the differentiation of U937d-, the cells were assayed for an induction in the activity of the terminal differentiation marker, non-specific esterase. This showed that treatment with the antisense oligonucleotide reproducibly resulted in approximately 50% of the cells having high levels of NSE activity. In contrast, only 2-3% of the cells treated with the sense or degenerate oligonucleotide showed induction of this marker. These findings therefore show that the block to differentiation in U937d- involves the

Figure 44

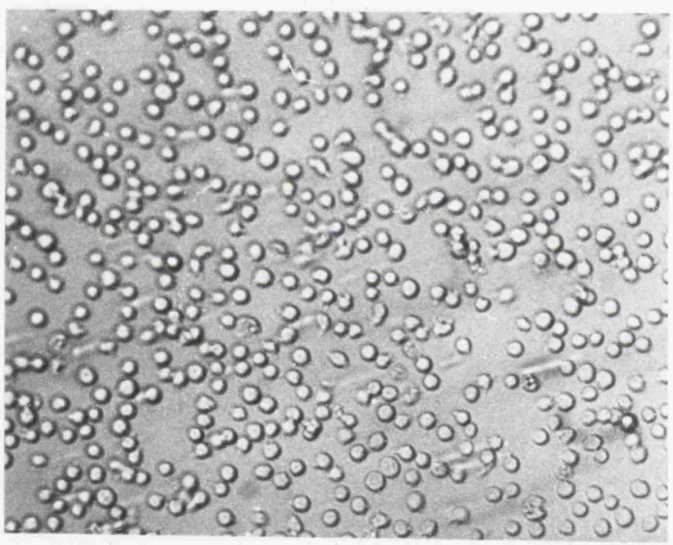
Figure 44: *Effect of c-myc antisense oligonucleotides on the differentiation capacity of TPA-treated U937d- cells.*

Morphological appearance of U937d- cells that have been treated with anti-sense oligonucleotides directed against the c-*myc* mRNA. Cells were treated for 5 days with TPA at a final concentration of 1.6 x x$10^{-7}$M and, where indicated, for 5 days with a daily addition of 20μM oligonucleotide. These effects were reproducibly seen in three independent experiments.

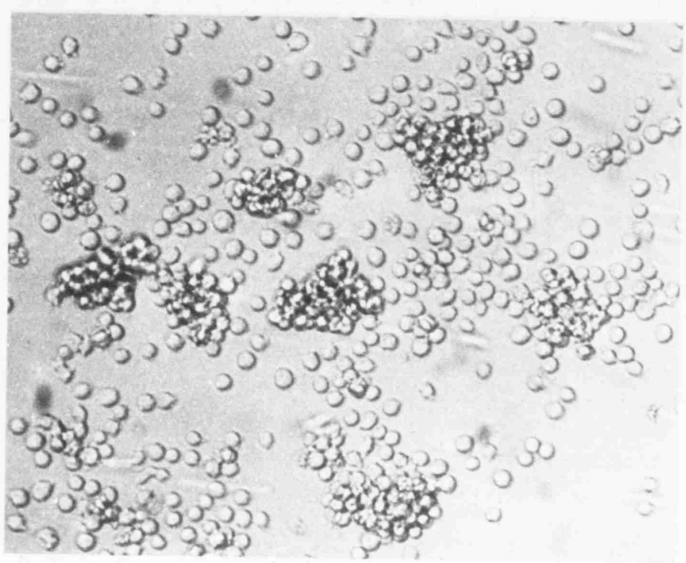
U937d-
+TPA



U937d-
+TPA
+ *myc* sense oligo



U937d-
+TPA
+ *myc* anti-sense oligo



continued expression of c-Myc protein (Figure 38), but does not involve the protein product of the Myc target gene, *ODC* (Figure 41). Moreover, coupled with the fact that this differentiation blockage is evident when U937d- cells are growth arrested, it must be the case that c-Myc affects this differentiation programme by a mechanism dissociated from its capacity to direct cell-cycle progression.

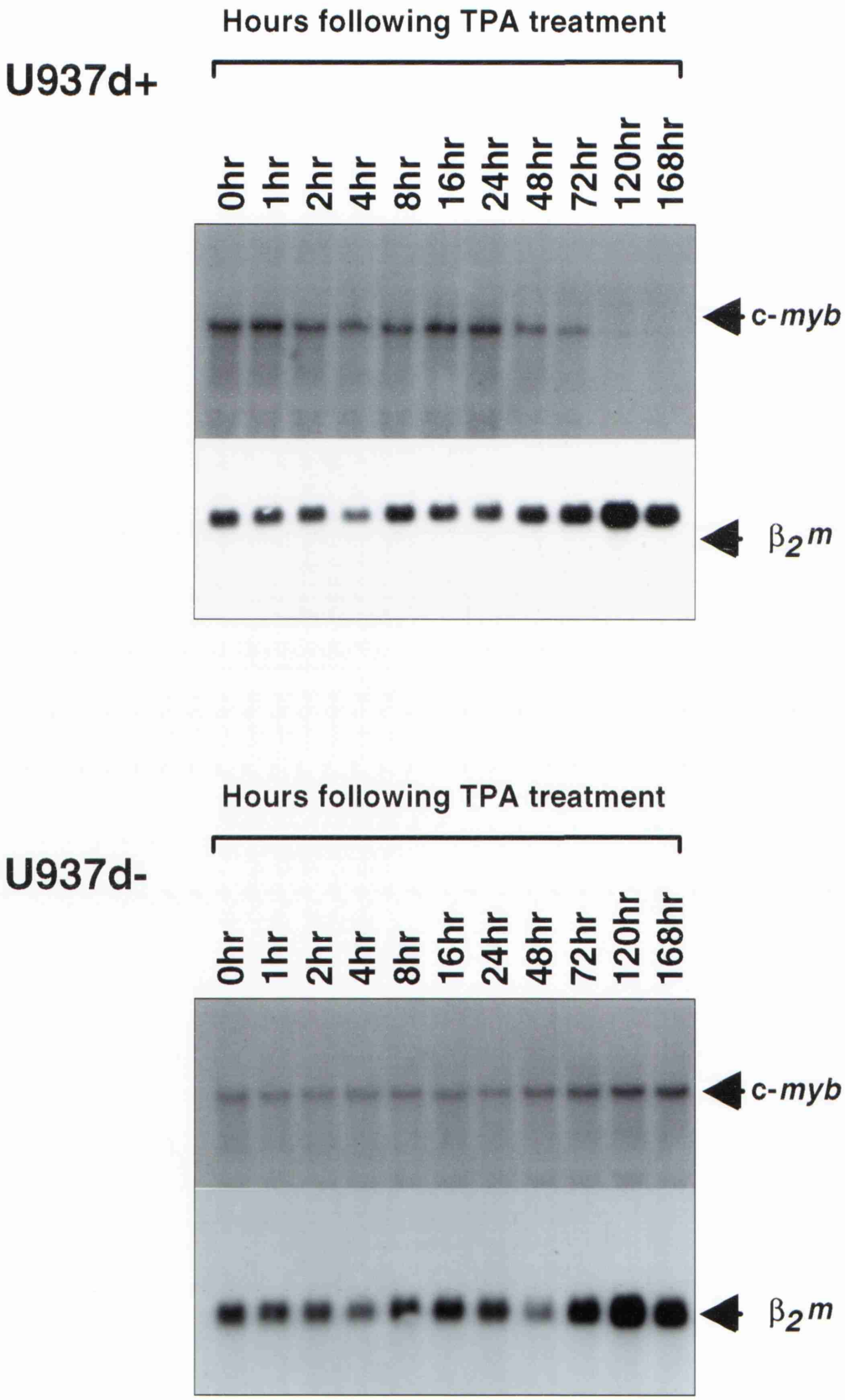
## 8.6 *Analysis of the changes in the levels of c-myb mRNA following TPA treatment of U937d+ and U937d- cells.*

During the comparison of the changes that occurred following TPA treatment of HL60 and HL60-Ast4 cells, it was an interesting observation that not only were the levels of c-*myc* mRNA regulated differently in the two cell lines (Figure 11 & 22), but so were the levels of c-*myb* mRNA (Figure 25). In light of this, it was decided to analyse the expression of c-*myb* following TPA treatment of U937d- cells. As can be seen in Figure 45, while the levels of c-*myb* mRNA are markedly decreased after 72hr of TPA treatment of U937d+ cells, the abundance of c-*myb* message in U937d- cells was not decreased and remained at least at pre-induction levels for the whole of the period analysed. When this observation is coupled with the findings in HL60-Ast4 cells it is indeed highly provocative. However, while it would have been interesting to have studied further the role of c-*myb*, particularly with respect to the role of c-*myc*, in the control of differentiation of these cells, this was made impossible due to the lack of suitable antibodies for the effective detection of c-Myb protein.

Figure 45

Figure 45: *Northern blot analysis of the levels of c-myb mRNA during TPA treatment of U937d+ and U937d- cells.*

Northern blot analysis of RNAs isolated, at the times indicated, from U937d+ and U937d- cells following treatment with TPA at a final concentration of $1.6 \times 10^{-7}$M. The blots was initially hybridised with a cDNA probe for c-*myb*, subsequently stripped and then as an assessment of loading were re-hybridised with a probe for $\beta_2$–*microglobulin.* Sections of the figure relating to each probing are labelled accordingly.


Hours following TPA treatment
U937d+
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
120hr
168hr
c-myb
β2m
Hours following TPA treatment
U937d-
0hr
1hr
2hr
4hr
8hr
16hr
24hr
48hr
72hr
120hr
168hr
c-myb
β2m

## CHAPTER 9: Mutational analysis of the genes encoding Max, Mad and Mxi1

### 9.1 *Southern blot analysis of c-myc, max, mad and mxi1 in a range of both myeloid and lymphoid leukaemias.*

As described previously, it is thought that changes in the levels and/or activities of Mad, Max and Mxi1 can have a bearing on the activity of c-Myc and, therefore, on the levels of c-Myc's target genes (Blackwood and Eisenman 1991; Ayer et al. 1993; Zervos et al. 1993). However, although there are many reports which implicate an involvement of c-Myc in many forms of malignant disease (Field and Spandidos 1990; Zimmerman and Alt 1990), there is little evidence to indicate that during this process, c-Myc activity might be affected by mutation of *max*, *mad* and *mxi1*. In order to explore this possibility, and with particular interest being given to the role of these proteins in haemopoietic differentiation, an analysis was made of the DNA from a range of myeloid and lymphoid leukaemias.

In order to increase the potentiality of finding mutations in *max*, *mad* and *mxi1*, samples of leukaemic DNAs were taken from Group stocks, which had previously not been shown to contain either amplifications and/or re-arrangements of the c-*myc* gene (Beatson Institute, Research Group 3; unpublished observations). In order to confirm that this was the case, Southern blots of these DNAs, together with DNAs from normal blood samples, were first hybridised with a probe corresponding to the third exon of the c-*myc* gene (see Methods 5.5.1). Indeed, as can be seen in Figure 46, when the levels of c-*myc* DNA is compared with that of $\beta_2$-*microglobulin*, which was used as a loading control, it is evident that these samples do not contain amplifications or gross re-arrangements at the c-*myc* locus.

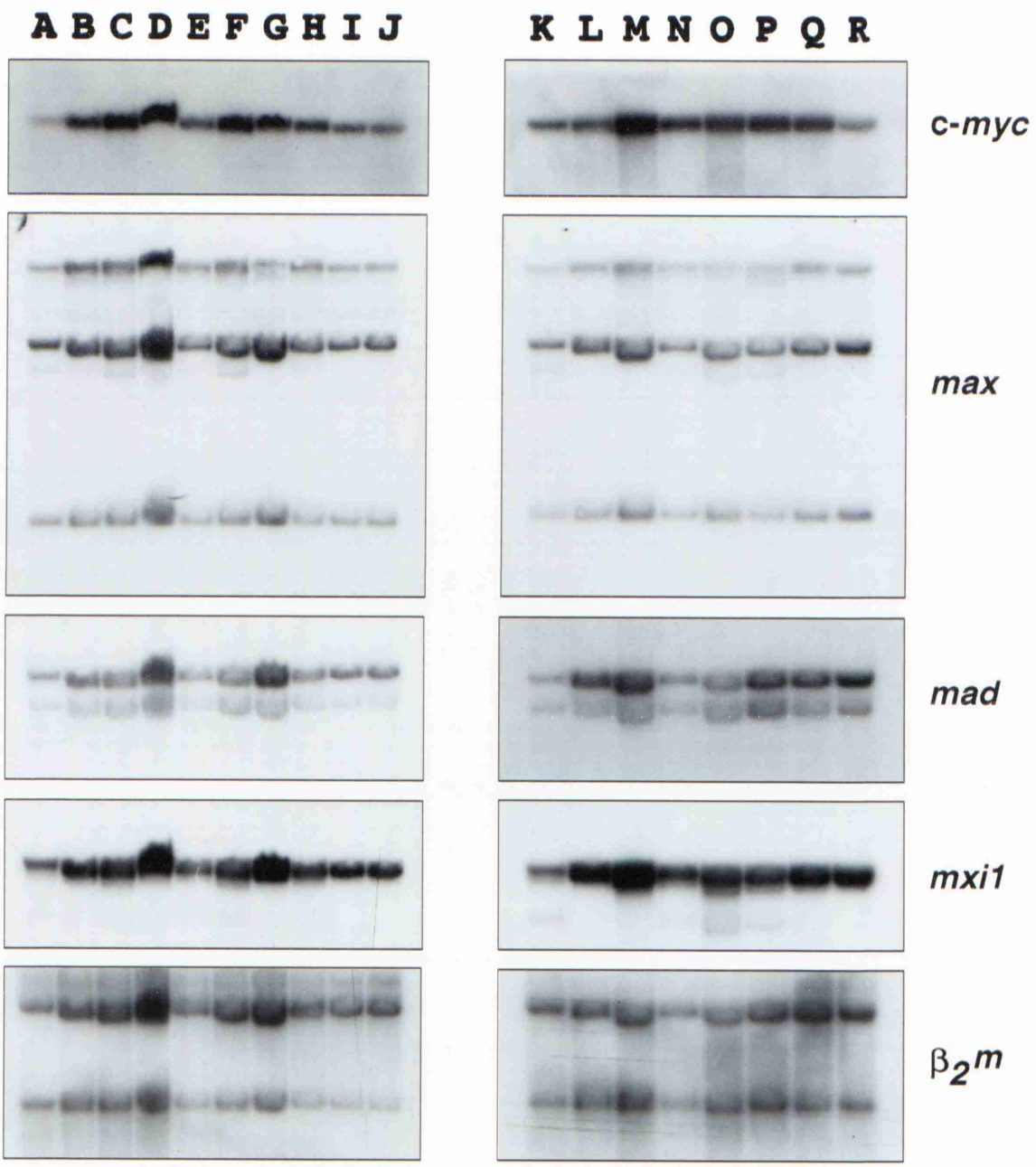
Figure 46

Figure 46: *Southern blot analysis of c-myc, max, mad and mxi1 in a range of myeloid and lymphoid leukaemias.*

DNAs were from the following sources:

| Lane | Source |
|---|---|
| A. | HFF cells. |
| B. | Normal white blood cells. |
| C. | Normal lymphocytes. |
| D. | Normal lymphocytes. |
| E. | Normal lymphocytes. |
| F. | K562 cells. |
| G. | Chronic granulocytic leukaemia (blast crisis). |
| H. | Acute myeloid leukaemia. |
| I. | Acute myeloid leukaemia. |
| J. | Acute myeloid leukaemia. |
| K. | Acute myeloid leukaemia. |
| L. | Acute myeloid leukaemia. |
| M. | Acute myeloid leukaemia. |
| N. | Acute myeloid leukaemia. |
| O. | Chronic lymphoblastic leukaemia. |
| P. | *null*-Acute lymphoblastic leukaemia. |
| Q. | Acute lymphoblastic leukaemia. |
| R. | Acute lymphoblastic leukaemia. |

DNA was digested with Eco RI and then transferred to Nylon membranes which were sequentially hybridised with probes for c-*myc*, *max*, *mad*, *mxi1* and $\beta_2$-*microglobulin* (see Materials and Methods for definition of probes).


A B C D E F G H I J
K L M N O P Q R
c-myc
max
mad
mxi1
$\beta_2 m$

As Mad, Max and Mxi1 have all been shown to be antagonists of c-Myc (Amin et al. 1993; Lahoz et al. 1994), it is predictable that any tumour-associated mutation in the genes encoding these proteins, that is detectable by Southern blotting, is most likely to be a deletion or an inactivating re-arrangement. However, when the Southern blots were hybridised with probes for these genes, not only were no deletions or re-arrangements observed, but there appeared to be no differences whatsoever between the normal and leukaemic samples analysed (Figure 46). Indeed, although this indicates that mutation of these genes at the macroscopic level was not involved in the establishment of the leukaemias analysed, it remains possible that the activities of the gene products may have been affected through point mutation. In addition, since only a small number of samples were analysed, this finding is not sufficient evidence to discount the possibility that these genes may well be involved in the establishment of other leukaemias or tumours of an unrelated nature.

9.2 *Analysis of the gene encoding Max in HeLa and HL60 cells.*

In addition to the study involving a range of leukaemias, the gene encoding Max was also analysed in HL60 cells. Southern blots were prepared to compare the amount of *max* DNA in HL60 cells to that in HeLa cells and primary human foreskin fibroblasts (HFF) cells (these cells were taken as a control of normal gene dosage). Surprisingly, when the amount of *max* DNA in each of the three samples was normalised against the levels of $\beta_2$-*microglobulin*, this appeared to show that HL60 cells, and possibly also HeLa cells, had a lower gene dosage than the primary HFF cells (Figure 47A). Indeed, when the blots were quantified using scanning laser densitometry, it was found that both HeLa and HL60 had approximately half the amount of *max* DNA than that in HFF cells (Figure 47B).

Figure 47

Figure 47: *Southern blot analysis to investigate the relative amounts of max DNA in HFF, HeLa and HL60 cells. Loading was normalised against $\beta_2M$.*

Panel A: DNA was digested with Hind III and then transferred to nylon membranes which were then sequentially hybridised with probes for *max* and $\beta_2M$. Exposure of blots was undertaken using Kodak X-OMAT film at -70°C with intensifying screens.

Panel B: Densitometric analysis of the relative amounts of *max* DNA in HFF, HeLa and HL60. The values which were obtained from the analysis of the *max*-hybridising bands were normalised against the values obtained from the analysis of $\beta_2M$.

The decreased levels of *max* was also seen in two repeats of the Southern blot which were undertaken using two different control DNAs.

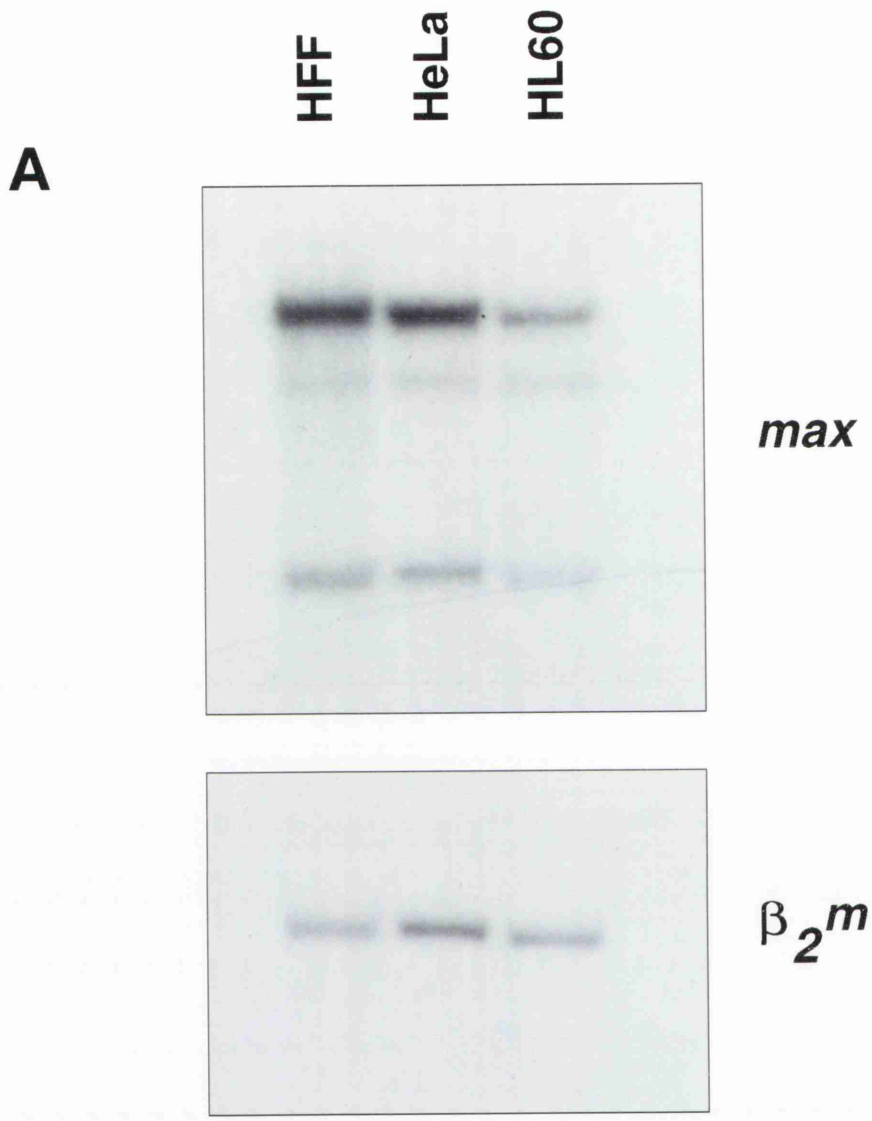

A
HFF
HeLa
HL60
max
β2m
B
Optical density x mm2 of max normalised against levels of β2m
14
12
10
8
6
4
2
0
HFF
HeLa
HL60

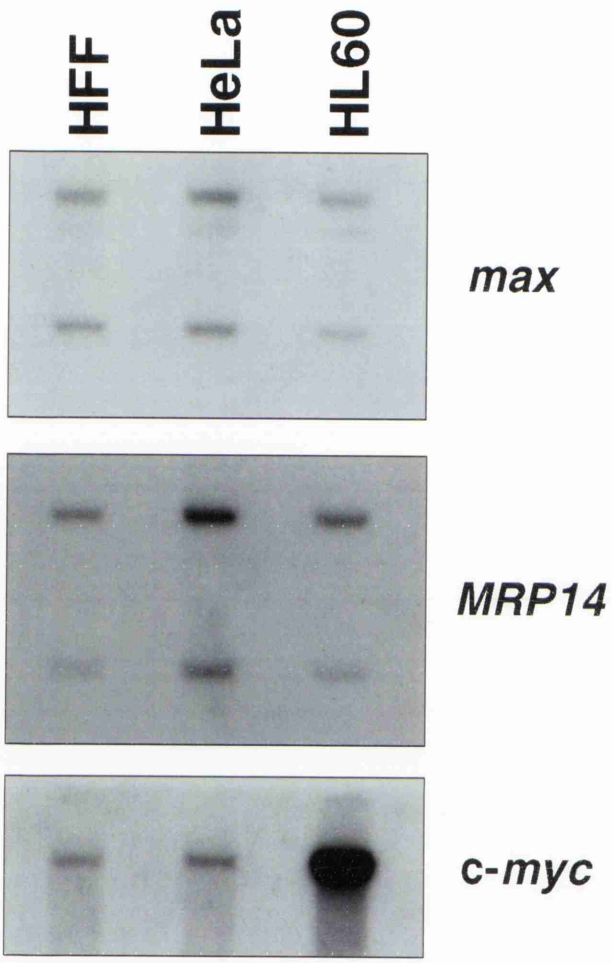
Figure 48

Figure 48: *Southern blot analysis to investigate the relative amounts of max DNA in HFF, HeLa and HL60 cells. Loading was normalised against $\beta_2M$.*

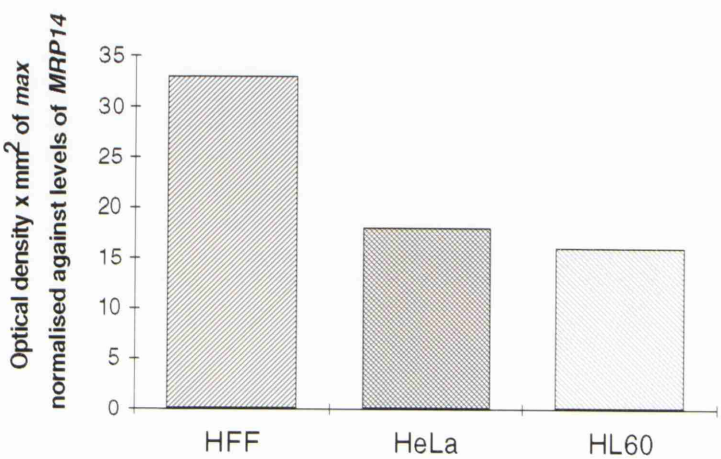
Panel A: DNA was digested with Hind III and then transferred to nylon membranes which were then sequentially hybridised with probes for *max* and $\beta_2M$. Exposure of blots was undertaken using Kodak X-OMAT film at -70°C without intensifying screens.

Panel B: Densitometric analysis of the relative amounts of max DNA in HFF, HeLa and HL60. The values which were obtained from the analysis of the *max*-hybridising bands were normalised against the values obtained from the analysis of *MRP14*.

A


HFF
HeLa
HL60
max
MRP14
c-myc

B


Optical density x mm2 of max
normalised against levels of MRP14
35
30
25
20
15
10
5
0
HFF
HeLa
HL60

It was initially considered that this observed difference in the amount of *max* DNA may be an artefact which had arisen through the non-linearity of X-ray film when used in conjunction with intensifying screens (Laskey 1984). In addition, it was also possible that the blots were being misinterpreted and it was not the amounts of max that was different in the three DNAs analysed, but in fact the amounts of $\beta_2$-*microglobulin*. To exclude these two possibilities, the Southern blot was repeated in the absence of intensifying screens and the amount of *max* DNA in each sample normalised against the gene encoding the calcium-binding protein, MRP14 (Lagasse and Clerc 1988). The MRP14 gene was particularly chosen as it is considered unlikely that it would be altered in the establishment of a transformed phenotype and because it is located on a different chromosome from $\beta_2$-*microglobulin* (data from Human Gene Mapping II). Indeed, as can be seen in Figure 48, this revealed that the observed difference in the gene dosage of *max* had not arisen through the two possible artefacts described above. Moreover, when the levels of *max* were assessed by densitometry and normalised against the levels of *MRP14*, it was again found that HeLa and HL60 cells contained approximately half the amount of DNA as HFF cells. Therefore, as it is known that the *max* gene is single copy (Wagner et al. 1992), these data clearly seem to indicate that *max* is reduced to hemizygosity in HeLa and HL60 cells.

## 9.3 *Sequencing of the coding region of the max mRNA from HFF, HeLa and HL60 cells.*

Since it is known that the correct functioning of c-Myc is dependent on dimerisation with Max, it was a surprise to find that the gene dosage of *max* is reduced in cells which are known to have high levels of c-*myc* mRNA (Graham et al. 1985; Birnie et al. 1986) and protein (Hann and Eisenman 1984). One possible reason for this, is that the putative remaining allele of *max* is mutated

Figure 49

Figure 49: *Comparison of the sequence of the coding region of max in HFF, HeLa and HL60 cells.*

The sequence of the *max* coding region in HFF, HeLa and HL60 cells was first determined by direct sequencing using 'Dynabeads' of cDNAs generated by RT-PCR from the respective mRNAs. This was then confirmed by the automated sequencing in both directions of cDNAs generated in a second round of RT-PCR reactions. The sequences are aligned against the published sequence (Blackwood and Eisenman 1991), and nucleotides which were found to be different from this sequence are shown in bold. The stop codon is depicted as *.

**Pub.Seq** **A)** atg agc gat aac gat gac atc gag gtg gag agc

**HFF** **B)** atg agc gat aac gat gac atc gag gtg gag agc

**HeLa** **C)** atg agc gat aac gat gac atc gag gtg gag agc

**HL60** **D)** atg agc gat aac gat gac atc gag gtg gag agc

A) gac gct gac aaa cgg gct cat cat aat gca ctg gaa cga aaa
B) gac gct gac aaa cgg gct cat cat aat gca ctg gaa cga aaa
C) gac gct gac aaa cgg gct cat cat aat gca ctg gaa cga aaa
D) gac gct gac aaa cgg gct cat cat aat gca ctg gaa cga aaa

A) cgt agg gac cac atc aaa gac agc ttt cac agt ttg cgg gac
B) cgt agg gac cac atc aaa gac agc ttt cac agt ttg cgg gac
C) cgt agg gac cac atc aaa gac agc ttt cac agt ttg cgg gac
D) cgt agg gac cac atc aaa gac agc ttt cac agt ttg cgg gac

A) tca gtc cca tca ctc caa gga gag aag gca tcc cgg gcc caa
B) tca gtc cca tca ctc caa gga gag aag gca tcc cgg gcc caa
C) tca gtc cca tca ctc caa gga gag aag gca tcc cgg gcc caa
D) tca gtc cca tca ctc caa gga gag aag gca tcc cgg gcc caa

A) atg cta gac aaa gcc aca gag tat atc cag tat atg cga agg
B) atg cta gac aaa gcc aca gaa tat atc cag tat atg cga agg
C) atg cta gac aaa gcc aca gaa tat atc cag tat atg cga agg
D) atg cta gac aaa gcc aca gaa tat atc cag tat atg cga agg

A) aaa aac cac aca cac cag caa gat att gac gac ctc aag cgg
B) aaa aac cac aca cac cag caa gat att gac gac ctc aag cgg
C) aaa aac cac aca cac cag caa gat att gac gac ctc aag cgg
D) aaa aac cac aca cac cag caa gat att gac gac ctc aag cgg

A) cag aat gct ctt ctg gag cag caa gtc cgt gca ctg gag aag
B) cag aat gct ctt ctg gag cag caa gtc cgt gca ctg gag aag
C) cag aat gct ctt ctg gag cag caa gtc cgt gca ctg gag aag
D) cag aat gct ctt ctg gag cag caa gtc cgt gca ctg gag aag

A) gcg agg tca agt gcc caa ctg gag acc aac tac ccc tcc tca
B) gcg agg tca agt gcc caa ctg gag acc aac tac ccc tcc tca
C) gcg agg tca agt gcc caa ctg gag acc aac tac ccc tcc tca
D) gcg agg tca agt gcc caa ctg gag acc aac tac ccc tcc tca

A) gac aac agc ctc tac acc aac gcc aag ggc agc acc atc tct
B) gac aac agc ctc tac acc aac gcc aag ggc agc acc atc tct
C) gac aac agc ctc tac acc aac gcc aag ggc agc acc atc tct
D) gac aac agc ctc tac acc aac gcc aag ggc agc acc atc tct

A) gcc ttc gat ggg ggc tca gac tcc agc tca gag tct gag cct
B) gcc ttc gat ggg ggc tcg gac tcc agc tcg gag tct gag cct
C) gcc ttc gat ggg ggc tcg gac tcc agc tcg gag tct gag cct
D) gcc ttc gat ggg ggc tcg gac tcc agc tcg gag tct gag cct

A) gaa gag ccc caa agc agg aag aag ctc cgg atg gag gcc agc *
B) gaa gag ccc caa agc agg aag aag ctc cgg atg gag gcc agc *
C) gaa gag ccc caa agc agg aag aag ctc cgg atg gag gcc agc *
D) gaa gag ccc caa agc agg aag aag ctc cgg atg gag gcc agc *

in such a way to either facilitate the functioning of c-Myc or to impede the function of a Max-dependent Myc-antagonist, for example, Mad or Mxi1. To investigate this, the coding region of *max* was amplified by RT-PCR from RNA derived from HFF, HeLa and HL60 cells. The resulting product was then initially sequenced using 'Dynabeads' (see Methods 5.4.3) and then the result obtained confirmed by sequencing in both directions using the Institute's Automated Sequencer Facility. Although somewhat disappointing with respect to the initial hypothesis, when the sequence of *max* in HFF, HeLa and HL60 cells were compared, it was found that they were in fact identical (Figure 49). Nevertheless, when these sequences were compared to the published sequence, which was from the Manca cell line (Blackwood and Eisenman 1991), three base pair substitutions were observed (Figure 49). However, it was again disappointing to find that, each of these substitutions occurred at the third positions of codons and in these particular cases did not result in an amino acid change (i.e. gag (Glu)→ gaa (Glu); tca (Ser)→ tcg (Ser)). It seems likely therefore, that these changes do not represent genuine mutations in the Manca cell line, and are most probably either polymorphisms or sequencing errors in the original article relating to the cloning of the *max* cDNA (Blackwood and Eisenman 1991). However, re-sequencing of the coding region of *max* in the Manca cell line would be required to confirm that this were the case.

# DISCUSSION

# CHAPTER 10: DISCUSSION.

## 10.1 *A role for Mad, Max and Mxi1 in processes of myeloid differentiation?*

In the initial stages of this study an analysis was undertaken to gain insight into the involvement of Max, Mad and Mxi1 in processes of myeloid differentiation. Moreover, as it became clear as each of these proteins was discovered that they can all having a bearing on c-Myc function (Amin et al. 1993; Lahoz et al. 1994), particular interest was also given to they way in which any changes in their abundance or activity may also affect c-Myc. To this end, an analysis was made of the relative levels of the mRNAs for these proteins during the induced differentiation of myeloid leukaemic HL60 and U937 cells. As *mad* and *mxi1* were only discovered after the instigation of this work, this study was initially undertaken on *max*. This revealed that, in contrast to the rapid decrease in c-*myc* mRNA that occurs following differentiation in HL60 cells induced by treatment with TPA and DMSO, the abundance of *max* mRNA, despite some moderate fluctuations remained at levels comparable to those observed in untreated cells for the majority of the differentiation programmes. However, it was apparent that the abundance of the mRNA was markedly decreased (~9-fold) at the very late stages of differentiation to monocytes/macrophages (induced by TPA). As it is now known Max protein is essential for the correct functioning of not just Myc, but also Mad and Mxi1 (Blackwood and Eisenman 1991; Ayer et al. 1993; Zervos et al. 1993) it is perhaps no surprise that the mRNA is found at a relatively consistent level for the majority of both these differentiation programmes. However, why its abundance is decreased at the late stages of differentiation to monocytes/macrophages is more difficult to explain. It might initially be considered that, as this decrease was not observed following differentiation in HL60 cells induced by Vit D3, and that only a very small decrease in *max* mRNA occurred during TPA-induced differentiation in U937 cells,

this effect might reflect an inducer or cell-type-specific phenomenon. However, during the course of this work, the levels of *max* mRNA and protein were independently also found to undergo a considerable decrease during differentiation of MEL cells induced by N,N'-hexamethylene bisacetamide (Dunn et al. 1994). Taken together these findings indicate that this decrease in the levels of *max* mRNA could well be a facet of differentiation in certain situations and may therefore be worthy of further study. However, since this change occurs so late in the differentiation programme, it seems extremely unlikely that any inferred decline in the abundance of Max protein will have any consequence for c-Myc function.

The subsequent analysis of *mxi1* mRNA during differentiation of both HL60 and U937 cells, revealed that the levels of the message were comparable to those in untreated cells at all of the time points studied. This finding is in agreement with an independent study that was published during the course of this work (Larsson et al. 1994), but is in contrast to what was reported in the article relating to the original cloning of this gene (Zervos et al. 1993). As part of the latter study, it was found that the levels of *mxi1* mRNA are extremely low in untreated U937 cells, but undergo a considerable increase following induction of differentiation by treatment with TPA. One possible explanation for this discrepancy is that this reflects a clonal difference between the cells used by Zervos et al. (1993) and those used in this study or by Larsson et al. (1994). In this regard, it is noteworthy that, in the study by Zervos et al. (1994), the levels of c-*myc* mRNA were also low in untreated cells and underwent a marked increase following treatment with TPA. Not only is this observation again in disagreement with what was observed in the study presented here and in the report by Larsson et al. (1994), it is also in contrast to previous studies of c-*myc* mRNA during differentiation in this system (Einat et al. 1985; Eickholt et al. 1993). However, the observations by Zervos et al. (1993) cannot be discounted, and as a result the only fair conclusion which can be drawn, is that the findings of the study presented here indicate that, due to the invariant levels

of *mxi1* mRNA, it seems unlikely that this gene has a central role in the control of differentiation in this lineage.

In agreement with the report by Larsson et al. (1994), analysis of *mad* mRNA in HL60 and U937 cells revealed that an increase in the abundance of the message was a rapid event during differentiation induced by TPA. When this observation is coupled with the finding that this increase can occur without new protein synthesis (Ayer and Eisenman 1993), it is possible that up-regulation of *mad* is a determining event during differentiation in these systems. In addition, as the increase in *mad* expression occurs prior to the loss of c-*myc* mRNA and protein that occurs during these processes, this opens up the possibility that the induction of *mad* expression is the initial and possible primary event involved in the abrogation of Myc:Max function during the differentiation of these cells. However, when further analysis was made of the expression patterns of *mad* during differentiation of the clone of HL60 cells used in the study presented here, it was found that differentiation to both granulocytes (using DMSO) and to monocytes/macrophages (using Vit D3) could also be achieved without a concomitant increase in the levels of *mad* mRNA. This therefore precludes the possibility that up-regulation of *mad* expression is an essential event for differentiation in these cells. In addition, this would also indicate that the up-regulation of *mad* is not a necessary process for the inactivation of the differentiation-blocking activity of c-Myc. It must be pointed out, however, that this does not rule out a role for the up-regulation of *mad* that was observed during the programme of differentiation induced by TPA. Furthermore, as HL60 cells, at all of the time points analysed, express a basal level of *mad* mRNA, these data cannot exclude a role for Mad protein during differentiation of these cells induced by either of the three agents used, whether in the regulation of Myc activity or in a non-Myc-related function.

To investigate further the role of *mad* during differentiation induced by TPA, analysis was made of a differentiation-defective variant of HL60 cells, HL60-

Ast4 (Bunce et al. 1983). This variant, which fails to differentiate in response to TPA, is similar to "wild-type" HL60 cells in that following treatment it undergoes a growth arrest in the $G_0/G_1$ phase of the cell cycle. It was therefore hypothesised, that if the observed increase in the levels of *mad* mRNA during TPA-induced differentiation of "wild-type" cells was intrinsically associated with processes of differentiation and not with other events that take place following treatment with TPA for example, cell-cycle arrest, then an increase in the abundance of *mad* mRNA should not occur following TPA treatment of Ast4 cells. However, analysis of the cells revealed that the abundance of *mad* mRNA did undergo a rapid increase following treatment with TPA and that these increased levels were then maintained for the rest of the period analysed. Upon this finding it was speculated that, not only could myeloid differentiation occur without a concomitant increase in the levels of *mad* mRNA (as was observed following differentiation induced by DMSO and Vit D3), but perhaps the abundance of *mad* mRNA could be elevated without an accompanying programme of differentiation. To this end, as it was known that TPA mediates at least some its functions by mimicking the effects of diacylglycerols on protein kinase C (PKC) (Nakiki et al. 1984), it was decided to treat U937 cells with the synthetic diacylglycerol, OAG (Kaibuchi et el. 1983). This cell line and agent were particularly chosen as it had previously been reported that despite activating PKC, OAG does not induce differentiation of U937 cells (Ways et al. 1987). In agreement with the initial hypothesis, it was found that the levels of *mad* mRNA were rapidly increased following treatment of U937 cells with this diacylglycerol. Therefore, when this finding is coupled with the fact that myeloid leukaemia cells can be differentiated without a concomitant increase in the levels of *mad* mRNA, this would seem to indicate that an elevation of *mad* mRNA abundance is neither sufficient nor obligatory for differentiation in these systems. However, it must be pointed out, that as the OAG-induced increase in *mad* mRNA was different from that observed following treatment with TPA, in that it was only transient, these

findings cannot exclude the possibility that a sustained elevation of *mad* mRNA abundance may well be sufficient to instigate a programme of differentiation in these cells. In addition, these data can also not be taken to indicate that, in situations were *mad* mRNA is increased, its elevation is superfluous to the accompanying process of differentiation. In this regard, a noteworthy parallel has been reported for the c-*fos* proto-oncogene. It was initially reported that an increase in the levels of c-*fos* mRNA was a very early event which occurred during differentiation to monocytes/macrophages (Muller et al. 1984; Muller et al. 1985), but not during differentiation to granulocytes (Mitchell et al. 1985). In a similar manner to what was considered following the analysis of *mad* mRNA in the study presented here, these findings led to the speculation that c-*fos* played a pivotal role during differentiation of the myelomonocytic lineage. However, again in a similar way to what was found here in relation to *mad*, this notion was later revoked upon the finding that increased levels of c-*fos* was neither necessary nor adequate for efficient differentiation to monocytes/macrophages (Mitchell et al. 1986). It was only upon the disruption of c-*fos* in mice by homologous recombination that the true role of this gene was revealed. Although these mice survived to term, they were found to have multiple defects in the development of both bone and haemopoietic cells, including those of the myelomonocytic lineage (Wang et al. 1992). Therefore, as this is a clear indication of the importance of c-*fos* in these processes, perhaps the generation of mice which lack *mad* expression will also shed more light on the role played by this gene in the control of differentiation to monocytes/macrophages.

### 10.2 *Changes in E-box DNA-binding during myeloid differentiation.*

These initial studies were extended to gain greater insight as to the role of Mad within these systems. As it is known that c-Myc is a key regulator of the growth and differentiation of these cells, it is reasonable to assume that the

regulation of c-Myc's target genes (e.g. ornithine decarboxylase (Wagner et al. 1993) and α-prothymosin (Eilers et al. 1991)) is critical to the way in which c-Myc controls these processes. Since Mad:Max, Max:Max and Mxi1:Max dimers have all been shown to repress transcription through the same consensus DNA-binding site through which Myc:Max activates (CACGTG) (Ayer et al. 1993), and upon which the expression of ornithine decarboxylase and α-prothymosin has been shown to be dependent (Bello-Fernandez et al. 1993; Gaubatz et al. 1994), an analysis was made of the proteins which bind to this site during the induced differentiation of HL60 cells. These studies revealed that loss of Myc:Max DNA binding was an early event during differentiation down both pathways. Moreover, the profiles of Myc:Max complex levels were extremely similar to the changes in the levels of c-*myc* mRNA. In this regard, it is easy therefore to perceive how a signal which affects the abundance of c-*myc* mRNA could be rapidly translated into target gene activation and ultimately phenotypic change. It is perfectly understandable, therefore, why the levels and activity of c-Myc have been found to be subject to such intricate control (Marcu et al. 1992).

In contrast to what was observed for Myc:Max, it was found that no Mad-containing complexes were detectable during differentiation of the cells to monocytes/macrophages. In addition, those which were observed during the differentiation of the cells to granulocytes were only detected during the late stages of the differentiation programme. This therefore indicates that, since these profiles of DNA binding are in contrast to the observed changes in the levels of *mad* message, the regulation of Mad DNA binding during this process is not solely determined by the abundance of its mRNA. Moreover, as Mad DNA binding is only detectable subsequent to the down-regulation of the Myc:Max complex, it would appear that involvement of Mad as a primary factor in the abrogation of c-Myc function is very unlikely. However, it is impossible to discount that these Mad-

containing complexes may well have a bearing on the differentiation process by a mechanism that is distinct from Mad's ability to antagonise the function of c-Myc.

The most surprising finding from these analyses was the observation that the Mad-containing complexes that were found during the differentiation to granulocytes did not contain Max. However, from the subsequent analysis of these complexes with three different antisera raised against different portions of the Max protein, it is clear that there is a genuine absence of Max within these DNA binding complexes. This finding is in contention with previous data that indicate that all Myc-related and Mad-related proteins are dependent on dimerization with Max in order to bind DNA effectively (Amati and Land 1994). After close analysis of the literature regarding Mad-containing complexes in cellular extracts it was found that they had only been detected in proteins which had previously been subject to immunoprecipitation with antisera raised against Max (Ayer and Eisenman 1993). Therefore, the detection of any complex which contained Mad, but not Max would not be possible under these conditions. Consequently, as the assay conducted here was undertaken using unfractionated nuclear proteins the observation of complexes that contain Mad but not Max, is not in conflict with the current status of knowledge regarding these proteins. More interestingly, since it is known that Mad cannot bind the CACGTG motif alone (Ayer et al. 1993), it must be that it is bound as part of a heterodimeric complex that does not contain Max. In light of this, the data presented here open up this transcription factor network to include proteins, which may or may not be related to Max, which can facilitate the binding of Mad in a sequence-specific manner.

### 10.3 *The mechanism of c-Myc-induced differentiation blockage.*

As the results gained from the initial stages of this study indicated that, Max, Mad and Mxi1 were unlikely to be determining factors in the differentiation of

this lineage, it was decided to change the focus of the investigation to analyse further the role played by c-Myc in these processes. In this regard, although it was clear that c-Myc was able to block the differentiation of a number of cell lineages (Freytag 1988; Miner and Wold 1988), including myeloid cells (Larsson et al. 1988), the mechanism of how c-Myc achieved this phenotypic effect remained ill-defined. As it was known that terminal differentiation in these cell types occurs simultaneously with a withdrawal from the cell cycle and down-regulation of the levels of c-Myc (Ingvarsson 1990; Zimmerman and Alt 1990), two schools of thought were generated as to how c-Myc might control this process. Firstly, constitutive expression of c-Myc could be interfering with the differentiation machinery by a mechanism distinct from its involvement in cell-cycle progression. Alternatively, it could be that by invoking cell-cycle progression, c-Myc could indirectly affect this process, as it is considered that, in general, proliferation and terminal differentiation are mutually exclusive. In order to address this question, it was decided to utilise a variant of the U937 leukaemic cell line, U937d-. This line is similar to HL60-Ast4 cells, but unlike its "wild-type" counterpart, U937d+, in that although it responds to treatment with the differentiation inducer, TPA, by undergoing a rapid and irreversible growth arrest, it does not concomitantly undergo a programme of differentiation. However, the line was more amenable to study than HL60 -Ast4 in that following the establishment of a growth arrest, it did not subsequently enter a programme of cell death.

The finding that the levels of c-Myc protein in U937d- cells appeared to be unaffected by treatment with TPA were in contrast to what was observed during the induced differentiation of the parental line, U937d+. In addition, this continued c-Myc expression was also in conflict with the view that c-Myc alone is sufficient to direct cell-cycle progression (Eilers et al. 1990). However, as it is known that cell-cycle progression in general (Pegg 1986), including that induced by c-Myc, is dependant on the activity of the Myc target gene, *ODC*, the observation that the

cells do not continue to express *ODC* serves as a reason for why U937d- cells can growth arrest even in the presence of high levels of c-Myc protein. Since ODC activity has also been shown to be essential for c-Myc-induced apoptosis (Packham and Cleveland 1994), this finding also explains why U937d- cells do not undergo programmed cell death following treatment with TPA. In addition, since c-Myc-induced apoptosis is also known to be dependent on the tumour suppressor protein, p53 (Hermeking and Eick 1994), the discovery that U937 cells are devoid of this protein (Danova et al. 1990) could also explain why apoptosis did not occur when putrescine was used to substitute for the down-regulation of *ODC*.

The continued expression of c-Myc in U937d- cells, without accompanying cell-cycle progression, resulted in the postulate that the block to differentiation of these cells was invoked by the Myc protein in a cell-cycle, and also ODC, independent manner. When the levels of c-Myc protein in U937d- cells were down-regulated by using antisense oligonucleotides, this indeed resulted in the cells acquiring a terminally differentiated phenotype. While this is the first observation that c-Myc can block differentiation by a mechanism dissociated from its ability to direct cell-cycle progression, it is not the first time that the phenotypic effects of c-Myc have been shown to arise through divergent mechanisms. La Rocca et al. (1994) examined the effects of a series of C-terminal truncations of the c-Myc protein for their ability to transform and/or block the differentiation of primary quail myoblasts. One of these mutants retained the capacity to transform these cells, but had no effect upon the ability of the cells to differentiate. However, as it is not known how the processes of cell-cycle progression in human myeloid cells relates to morphological transformation in quail myoblasts, it is impossible to predict how these findings might be related.

The only previous study which would indicate that c-Myc regulates the processes of cell-cycle progression and differentiation in myeloid cells by two divergent mechanisms has been presented by Luk et al. (1982). They reported that

the treatment of human leukaemic HL60 cells with the ODC inhibitor, DFMO, caused a withdrawal from the cell cycle, but did not affect the differentiation status of these cells. It has, however, been shown that the down-regulation of the levels of c-Myc in HL60 cells by antisense oligonucleotides results in their differentiation (Holt et al. 1988; Bacon and Wickstrom 1991). When considered together, these two findings indicate that c-Myc regulates this differentiation process via a mechanism which is not dependent on the activity of ODC.

If the block to differentiation of myeloid cells is neither cell-cycle nor ODC-dependent, how then might it be considered that c-Myc can bring about this phenotypic effect? Depicted in Figure 50 are two models which speculate as to how c-Myc can orchestrate the two processes individually. In scheme A, a simple explanation is envisaged. Here c-Myc works solely in conjunction with Max to regulate the expression of its target genes through the E-box motif, CACGTG. The way in which it independently regulates the processes of cell-cycle progression and differentiation is explained by having two sets of Myc target genes. One set of genes regulates cell-cycle progression and will therefore contain *ODC*. The other set (X genes), which is shown here to exclude *ODC*, regulates the control of differentiation. Indeed, in line with the ideas of Freytag (1988) which were presented in the Introduction (section 2.4.2), it is possible that the genes which control differentiation are still involved in the control of the cell cycle, but instead of stimulating cell-cycle progression, they regulate entry into the distinct predifferentiation state, $G_D$. However, as $G_D$ is at present a totally hypothetical state, it is difficult to predict which genes it might involve. A more appealing possibility is that set X might contain genes which are involved in the establishment of a differentiated phenotype and which are repressed by high levels of c-Myc protein. One interesting gene which falls into this category is the adipogenic transcription factor C/EBPα (Samuelsson et al. 1991; Yeh and McKnight 1995). Not only has c-Myc been shown to repress the activity of this gene's promoter (Li

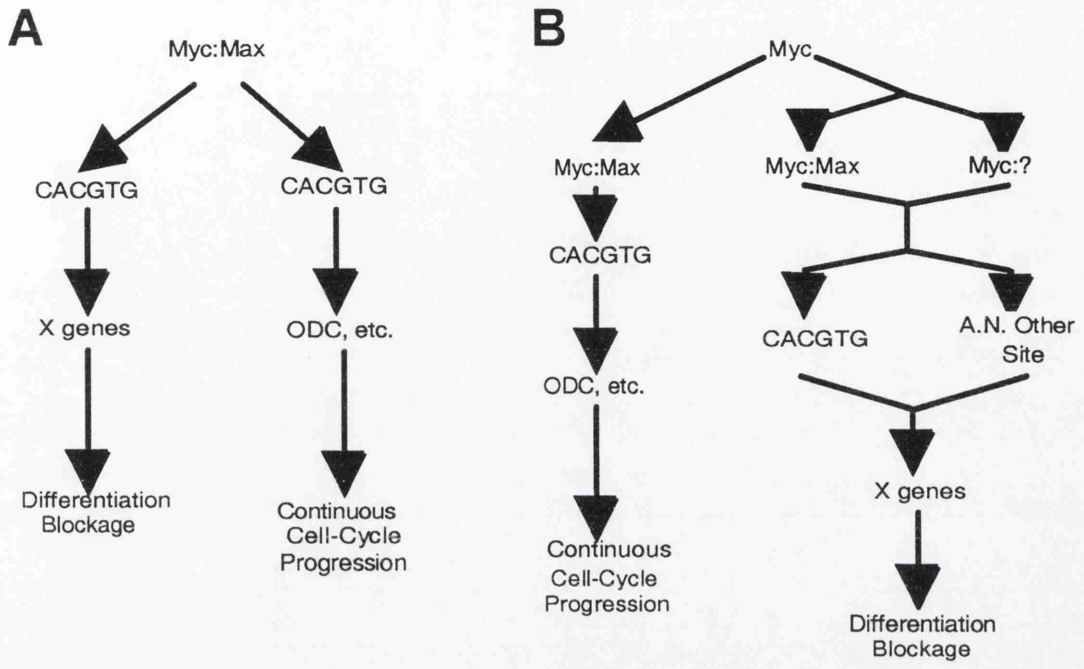



Figure 50: *Speculative mechanisms of Myc-induced differentiation blockage.*

In scheme A it is perceived that the control of cell-cycle progression and the regulation of differentiation are simply achieved by having two sets of Myc target genes, which are both regulated by Myc:Max through the E-box motif (CACGTG). Scheme B is more complicated, as it considers that the control of differentiation may not involve Max or the E-box and might involve other as yet unknown factors. See text for details..

et al. 1994), but enforced expression of C/EBPα has been shown to overcome the differentiation blockage induced by c-Myc in the pre-adipocyte cell line, 3T3-L1 (Freytag and Geddes 1992).

An alternative, more complicated, model to explain c-Myc's effects on differentiation is represented in scheme B. In this case, as it has been shown that the ability of c-Myc to affect cell-cycle progression is dependent not only on the activity of ODC, but also on dimerization with Max (Amati et al. 1993b), the pathway to cell-cycle progression remains the same as in scheme A. However, since there is no evidence that c-Myc requires dimerization with Max in order to block differentiation, it is possible that Myc forms a complex with a different protein to regulate the set of genes X. In this respect, it is pertinent that, while c-Myc has been shown to block the differentiation of the nerve growth factor-responsive PC12 cell line (Maruyama et al. 1987), it has recently been reported that these cells are devoid of functional Max protein (Hopewell and Ziff 1995). A number of other proteins have now been shown to interact with c-Myc (e.g. the transcription factors YY-1 (Shrivastava et al. 1993) and TFII-I (Roy et al. 1993) and the Rb-related protein, p107 (Beijersbergen et al. 1994; Gu et al. 1994)). However, the way in which any of these might affect c-Myc's ability to affect the differentiation process is yet to be determined.

The model in scheme B is further complicated by the consideration that the regulation of the differentiation-blocking genes X may not occur via c-Myc binding to the E-box motif, CACGTG. The finding that the larger translation product of c-*myc* can, in conjunction with Max, activate transcription through the binding sites of the C/EBP family of transcription factors (Hann et al. 1994) may be one way in which this could occur. Alternatively, by a Max-independent mechanism, it has been shown that c-Myc. can repress transcription through the Initiator element (Inr) (Roy et al. 1993). In this regard, it is noteworthy that the

repression of the C/EBPα gene by c-Myc has been shown to be dependent on the integrity of the Inr element within this gene's promoter (Li et al. 1994) .

It must finally be considered how these findings could relate to the establishment of neoplasia *in vivo*. A role in the genesis of leukaemia is particularly relevant since this disease has become a paradigm for tumours in which a differentiation blockage is considered to be involved in the establishment of the fully malignant state (Sawyers et al. 1991). In addition, mutation of the c-*myc* gene and/or elevated levels of c-*myc* mRNA and protein have been shown to be characteristic events in the blast cell populations of some leukaemic patients (Birnie et al. 1986). In a similar manner to the situation described for differentiation *in vitro*, c-Myc over-expression could give rise to a leukaemia, either directly through its effects on cellular proliferation or by affecting the differentiation process *per se*. In the latter regard, it can also quite easily be perceived how c-Myc, by solely affecting the differentiation process, could in turn affect cellular proliferation by restricting cells to a haemopoietic compartment in which proliferation is a continuous process. However, as at this stage very little is known about the differentiation of these cells *in vivo*, more work is required to fully understand the relationship of c-Myc to the many processes within this system.

## 10.4 *A role for c-myb in the regulation of c-myc and the control of differentiation?*

In addition to the differences observed for c-*myc* mRNA and protein, it was also highly provocative to find that the levels of c-*myb* mRNA were regulated differently in HL60-Ast4 cells and U937d- cells, than in their normal counterparts. As it has been proposed that c-*myc* is a target for c-*myb* transactivation (Nakagoshi et al. 1992), it is possible that these observations from the analysis of the differentiation-defective cell lines are indeed connected. In this regard, it might

therefore be considered that as the c-*myc* mRNA has been shown to be very unstable (Dani et al. 1984), any changes in the abundance of the c-*myb* mRNA will be followed by a corresponding change in the levels of the c-*myc* message. That is of course provided that the transactivation potential of c-*myb* is proportional to the abundance of its mRNA. In line with this notion, analysis of HL60-Ast4 revealed that a transient decrease in the levels of c-*myb* mRNA, which occurred following treatment of the cells with TPA, was subsequently mirrored by a slightly later transient decrease in the abundance of c-*myc* message. However, in contrast, although a transient decrease in the levels of c-*myc* mRNA was also observed following TPA treatment of U937d- cells, no such change was observed upon analysis of c-*myb* message. Of course, although this finding is in conflict with the idea that c-*myb* might block differentiation by transactivation of c-*myc*, it would be naive to assume that during this process, the regulation of c-*myc* mRNA levels was solely determined by products of the c-*myb* gene. Further studies are therefore required not only to determine the role of c-*myb* in the control of c-*myc* expression, but also to discover the way in which the products of the two genes are related both to each other and to the regulation of differentiation in this lineage.

### 10.5 *Mutational analysis of mad, max and mxi1.*

Although the mutational analysis undertaken in his study was limited, it was an intriguing find that the *max* gene appeared to be reduced to hemizygosity in HeLa and HL60 cells. However, as the subsequent sequencing of the putative remaining allele did not reveal any mutations, it is difficult to predict the relevance of this change to the establishment of the phenotype of these cells. In this regard, it does indeed still remain possible that this reduction in gene dosage might affect the phenotype of the cells, without mutation, by causing a reduction in the cellular pool of Max protein. However, as it is now impossible to obtain normal material from

the specific tissue and patient from which these cell lines were derived, whether this possibility is correct will remain unknown.

It might be cynically considered that this reduction in the gene dosage of *max* is in no way involved in the phenotype on these cells, and has arisen either through a deletion which involves a tumour suppressor gene which maps close to *max* or even merely as a result of the increased genome instability that is evident in transformed cells. However, alterations at the *max* locus have now also been detected in PC12 cells which result in the total absence of the full-length Max protein (Hopewell and Ziff 1995). It might therefore now be pertinent to make a more extensive search for alterations in *max* in both transformed cell lines and *de novo* tumour material, comparing tumour with normal from the same patient.

### 10.6 *Future Prospects.*

It is clear that this work has brought to light some important issues which are worthy of further study. Firstly, despite an extensive analysis of the role of *max*, *mad* and *mxi1* in processes of myeloid differentiation, the true function of the protein products of these genes still remains unknown. In this regard, the generation of myeloid cell lines which contain these genes under the control of both constitutive and conditional promoters should serve to shed more light on this issue. In addition, as it was found that Mad can bind DNA in conjunction with factors other than Max, the identification of these factors would be particularly rewarding.

Although the analysis of the c-Myc protein in the U937d+ and U937d- cell lines yielded information which indicated that c-Myc's ability to negatively regulate differentiation can be dissociated from its ability to direct cell-cycle progression, the mechanism involved in mediating these two c-Myc functions is yet to be determined. However, the identification of further c-Myc target genes, and in particular those

which can be specifically associated with only a sub-set of c-Myc's ascribed functions, is almost certainly required before this can be fully achieved.

Lastly, as it is perceived that *max*, *mad* and *mxi1* may all function as tumour suppressor genes, the findings presented here that the *max* locus is likely to be hemizygous in HL60 and HeLa cells add weight to the reports that *max* and *mxi1*, respectively, are mutated in PC12 cells and some prostate cancers (Hopewell and Ziff 1995; Eagle et al. 1995). The time may therefore be right, to analyse the extent to which these genes may be contributing factors in the establishment of both prostate and other types of human malignancy.

# REFERENCES

## REFERENCES.


Adams, J.M., Gerondakis, S., Webb, E., Corcoran, L.M. and Cory, S. (1983) Cellular *myc* oncogene is altered by chromosomal translocation to an immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphoma. *Proc. Natl. Acad. Sci.* USA **80**, 1982-1986.

Adams, J.M., Harris, A.W., Pinkert, C.A., Corcoran, L.M., Alexander, W.S., Cory, S., Palmiter, R.D. and Brinster, R.L. (1985) The c-*myc* oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* **318**, 533-538.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1989) Molecular biology of the cell. 2nd ed., Garland Publishing, Inc.

Alexander, W.S., Schrader, J.W. and Adams, J.M. (1987) Expression of the c-*myc* oncogene under the control of the immunoglobulin enhancer in Eμ-*myc* transgenic mice. *Mol Cell Biol.* 7, 1436-1444.

Amati, B., Brooks, M.W., Levy, N., Littlewood, T.D., Evan, G.I. and Land, H. (1993a) Oncogenic activity of the c-Myc protein requires dimerization with Max. *Cell* **72**, 233-245.

Amati, B., Dalton, S., Brooks, M.W., Littlewood, T.D., Evan, G.I. and Land, H. (1992) Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max. *Nature* **359**,423-426.

Amati, B., Littlewood, T.D., Evan, G.I. and Land, H. (1993b) The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max. *EMBO J.* **12**, 5083-5087.

Amati. B. and Land, H. (1994) Myc-Max-Mad: a transcription factor network controlling cell cycle progression, differentiation and death. *Curr. Opin. Genet. Dev.* **4**, 102-108.

Amin, C., Wagner, A.J. and Hay, N. (1993) Sequence specific transcriptional activation by Myc and repression by Max. *Mol. Cell. Biol.* **13**, 383-390.

Anderson, N.L., Gemmell, M.A., Coussens, P.M., Maurao, S.-I. and Huberman, E. (1985) Specific protein phosphorylation in human promyelocytic HL-60 cells susceptible or resistant to induction of cell differentiation by phorbol-12-myristate-13-acetate. *Cancer Res.* **45**, 4955-4962.

Andrews, N.C. and Faller, D.V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.* **19**, 2499.

Arnold, A., Kim, H.G., Gaz, R.D., Eddy, R.L., Fukushima, Y., Byers, M.G., Shows, T.B. and Kronenberg, H.M. (1989) Molecular cloning and chromosomal mapping of DNA rearranged with the parathyroid hormone gene in a parathyroid adenoma. *J. Clin. Invest.* **83**, 2034-2040.

Asai, A., Miyagi, Y., Sugiyama, A., Nagashima, Y., Kanemitsu, H., Obinata, M., Mishima, K. and Kuchino, Y. (1994) The S-Myc protein having the abaility to induce opoptosis is selectively expressed in rat embryo chondrocytes. *Oncogene* **9**, 2345-2352.

Askew, D.S., Ashmun, R.A., Simmons, B.C. and Cleveland, J.L. (1991) Constitutive c-*myc* expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* **6**, 1915-1922.

Auvinen, M., Paasinen, A., Andersson, L.C. and Holtta, E. (1992) Ornithine decarboxylase activity is critical for cell transformation. *Nature* **360**,355-358.

Ayer, D.A. and Eisenman, R.N. (1993) A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. *Genes Dev.* **7**, 2110-2119.

Ayer, D.E., Kretzner, L. and Eisenman, R.N. (1993) Mad: a heterodimeric partner for Max that antagonises Myc transcriptional activity. *Cell* **72**, 211-222.

Ayer, D.E., Lawrence, Q.A. and Eisenman, R.N. (1995) Mad:Max transcriptional repression is mediated by ternary complex-formation with mammalian homologs of yeast repressor Sin3. *Cell* **80**, 767-776.

Bacon, T.A. and Wickstrom E. (1991) Daily addition of an anti-c-*myc* oligomer induces granulocytic differentiation of human promyelocytic leukemia HL-60 cells in both serum-containing and serum-free media. *Oncogene Res.* **6**, 21-32.

Bar-Ner, M., Messing, L.T., Cultraro, C.M., Birrer, M.J. and Segal, S. (1992) Regions of c-Myc protein that are necessary for transformation are also required for inhibition of differentiation of murine erythroleukemia cells. *Cell. Growth Differ.* **3**, 183-190.

Bauknecht, T., Angel, P., Royer, H-D. and zur Hausen, H. (1992) Identification of a negative regulatory domain in the papillomavirus type 18 promoter: interaction with the transcriptional repressor YY1. *EMBO J.* **11**, 4607-4617.

Beckmann, H., Sou, L.-K. and Kadesh, T. (1990) TFE3: a helix-loop-helix protein that activates transcription through the immunoglobulin enhancer μE3 motif. *Genes Dev.* **4**, 167-179.

Beijersbergen, R.L., Hijmans, E.M., Zhu, L. and Bernards, R. (1994) Interaction of c-Myc with the pRb-related protein p107 results in the inactivation of c-Myc-mediated transactivation. *EMBO J.* **13**, 4080-4086.

Bello-Fernandez, C., Packham, G. and Cleveland, J.L. (1993) The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc. Natl. Acad. Sci. USA* **90**, 7804-7808.

Bentley, D.L. and Groudine, M. (1986) Novel promoter upstream of the human c-*myc* gene and regulation of c-*myc* expression in B-cell lymphomas. *Mol. Cell. Biol.* **6**, 3481-3489.

Bentley, D.L. and Groudine, M. (1986) A block to elongation is largely responsibel for decreased transcription of c-*myc* in differentiated HL60 cells. *Nature* **321**, 702-706.

Bentley, D.L. and Groudine, M. (1988) Sequence requirements for premature termination of transcription of the human c-*myc* gene. *Cell* **53**, 245-256.

Bernard, O., Cory, S., Gerondakis, S., Webb, E. and Adams J.M. (1983) Sequence of the murine and human cellular *myc* oncogenes and two modes of myc transcription resulting from chromosomal translocation in B lymphoid tumours. *EMBO J.* **2**, 2375-2383.

Birnie, G.D. (1988) The HL60 cell line: A model system for studying human myeloid cell differentiation. *Br. J. Cancer* **58**, Suppl. IX, 41-45.

Birnie, G.D., Warnock, A.M., Burns, J.H. and Clark, P. (1986) Expression of the *myc* gene locus in populations of leukocytes from leukaemia patients and normal individuals. *Leukaemia Res.* **10**, 515-526.

Bissonnette, R., Echeverri, F., Mahboubi, A., Green, D. (1992) Apoptotic cell death induced by c-*myc* is inhibited by *bcl*-2. *Nature* **359**, 552-554.

Blackwell, T.K., Huang, J., Ma, A., Kretzner, L., Alt, F.W., Eisenman, R.N. and Weintraub, H. (1993) Binding of Myc proteins to canonical and non-canonical DNA-sequences. *Mol. Cell. Biol.* **13**, 5216-5224.

Blackwood, E.M. and Eisenman, R.N. (1991) Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science* **251**, 1211-1217.

Blackwood, E.M., Kretzner, L. and Eisenman, R.N. (1992) Myc and Max function as a nucleoprotein complex. *Curr. Opin. Genet. Dev.* **2**, 227-235.

Bodrug, S.E., Warner. B.J., Bath, M.L., Lindeman, G.J., Harris, A.W. and Adams, J.M. (1994) *Cyclin* D1 transgene impedes lymphocyte maturation and collaborates in lymphomagenesis with the *myc* gene. *EMBO J.* **13**, 2124-2130.

Boise, L.H., Gorse, K.M. and Westin, E.H. (1992) Multiple mechanisms of regulation of the human c-*myb* gene during myelomonocytic differentiation. *Oncogene* 7, 1817-1825.

Bos, J.L., Toksoz, D., Marshall, C.J., ver Laandevries, M., Veeneman, G.H., van der Eb, A., van Boom, J.H., Janssen, J.W.G. and Steenvoorden, A.C.M. (1985) Amino acid substitution at codon 13 of the N-*ras* oncogene in human acute myeloid leukaemia. *Nature* **315**, 726-730.

Bos, J.L., ver Laandevries, M., Janssen, A.M., Veeneman, G.H., van Boom, J.H. and van der Eb, A. (1984) 3 different mutations in codon 61 of the human N-*ras* gene detected by synthetic oligonucleotide hybridisation. *Nucleic Acids Res.* **12**, 9155-9163.

Bowlin, T.L., McKnown, B.J. and Sunkara, P.S. (1986) Ornithine decarboxylase induction and polyamine biosynthesis are required for the growth of interleukin-2- and interleukin-3-dependent cell lines. *Cell. Immunol.* **98**, 341-350.

Breitman, T., Selonick, S. and Collins, S. (1980) Induction of differentiation of the human promyelocytic cell line (HL-60) by retinoic acid. *Proc. Natl. Acad. Sci.* USA 77, 2936-2940.

Brennan, J.K., Abboud, C.N., DiPersio, J.F., Barlow, G.H. and Lichtman, M.A. (1981) Autostimulation of growth by human myelogenous leukemia cells (HL-60) Blood 58, 803-812.

Bunce, C.M., Fisher, A.G., Toksoz, D. and Brown, G. (1983) Isolation and characterisation of dimetyl sulphoxide resistant variants from the human promyeloid cell line, HL60. *Exp. Hematol.* **11**, 828-833.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) Direct activation of calcium-activated, phospholipid-dependent protein-kinase by tumour promoring phorbol esters. *J. Biol. Chem.* **257**, 7847-7851.

Clarke, M.F., Kukowskalatallo, J.F., Westin, E., Smith, M. and Prochownik, E.V. (1988) Constitutive expression of a c-*myb* cDNA blocks Friend murine erythroleukemia cell differentiation. *Mol. Cell. Biol.* **8**, 884-892.

Cleary, M.L., Smith, S.D. and Skiar, J. (1986) Cloning and structural analysis of cDNAs for *bcl*-2 and a hybrid *bcl*-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* **47**, 19-28.

Clinton, M., Frangoulazaridis, M., Panneerselvam, C. and Horecker, B.L. (1989) Proyhtmosin α and prothymosin mRNA and polypeptide levels in rodent tissues. *Arch. Biochem. Biophys.* **269**, 256-267.

Collins, S.J. (1987) The HL60 promyelocytic leukemia cell line: proliferation, differentiation and cellular oncogene expression. *Blood* **70**, 1233-1244.

Collins, S.J., Gallo, R.C. and Gallagher, R.E. (1977) Continuous, growth and differentiation of human myeloid leukemia cells in suspension culture. *Nature* **270**, 347-349.

Collins, S.J. and Groudine, M. (1982) Amplification of endogenous *myc*-related DNA-sequences in a human myeloid leukaemia cell line. *Nature* **298**, 697-681.

Coppola, J.A. and Cole, M.D. (1986) Constitutive c-*myc* oncogene expression blocks mouse erythroleukaemia cell differentiation but not commitment. *Nature* **320**, 760-763.

Crouch, D.H., Fisher, F., Clark, W., Jayaraman, P-S., Coding, C.R. and Gillespie, D.A.F. (1993) Gene-regulatory properties of Myc helix-loop-helix/leucine zipper mutants; Max-dependent transcriptional activation in yeast correlates with transforming activity. *Oncogene* **8**, 1849-1855.

Cuypers, H.T., Selten, G., Quint, W., Zijlstra, M., Maandag, E.R., Boelens, W., van Wezenbeek, P., Melief, C. and Berns, A. (1984) Murine leukemia virus-induced T-cell lymphomagenesis: Integration of proviruses in a distinct chromosomal region. *Cell* **37**, 141-150.

Daksis, J.I., Lu, R.Y., Facchini, L.M., Markin, W.W. and Penn, L.J.Z. (1994) Myc induces *cyclin* D1 expression in the absence of *de novo* protein synthesis and links mitogen-stimulated signal transduction to the cell cycle. *Oncogene* **9**, 3635-3645.

Dalla-Favera, R., Wong-Staal, F. and Gallo, R.C. (1982) *onc* gene amplification in promyelocytic leukaemia cell line HL-60 and primary leukaemic cells of the same patient. *Nature* **299**, 61-63.

Dalla-Favera, R., Martinotti, S., Gallo, R.C., Erikson, J. and Croce, C.M. (1983) Translocations and rearrangements of the c-*myc* oncogene locus in human undifferentiated B-cell lymphomas. *Science* **219**, 963-967.

Dalton, W.T., Ahearn, M.J., McCredie, K.B., Freireich, E.J., Stass, S.A. and Trujillo, J.M. (1988) HL-60 cell line was derived from a patient with FAB-M2 and not FAB-M3. *Blood* **71**, 242-247.

Daly, G.Q. and Benneriah, Y. (1991) Implicating the *bcr/abl* gene in the pathogenesis of Philadelphia chromosome-positive human leukemia. *Adv. Cancer Res.* **57**, 151-184.

Dang, C.V. and Lee, W.M.F. (1988) Identification of the human c-Myc protein nuclear translocation signal. *Mol. Cell. Biol.* **8**, 4048-4054.

Dang, C.V., McGuire, M., Buckmire, M. and Lee W.M.F. (1989) Involvement of the leucine zipper region in the oligomerization and transforming activity of human c-Myc protein. *Nature* **337**, 664-666.

Dani, C., Blanchard, J-M., Piechaczyk, M., El Sabouty, S., Marty, L. and Jeanteur, P. (1984) Extreme instability of *myc* messanger RNA in normal and transformed human cells. *Proc. Natl. Acad. Sci.* USA **81**, 7046-7050.

Danova, M., Giordano, M., Mazzini, G. and Riccardi, A. (1990) Expression of p53 protein during the cell cycle measured by flow cytometry in human leukemia. *Leukemia Res.* **14**, 417-422.

Davies, R., Hicks, R., Crook, T., Morris, J. and Vousden, K. (1993) Human papillomavirus type 16 E7 associates with a histone H1 kinase and with p107 through sequences necessary for transformation. *J. Virol.* **67**, 2521-2528.

Davis, R.L., Cheng, P-F., Lassar, A.B. and Weintraub, H. (1990) The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell* **60**, 733-746.

Debbas, M. and White, E. (1993) Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev.* 7, 546-554.

Delarosa, A., Williams, R.L. and Steeg, P.S. (1995) NM23 nucleotide diphosphate kinase: towards a structural and biochemical understanding of its biological functions. *Bioessays* **17**, 53-62.

Dmitrovsky, E., Kuehl, W.M., Hollis, G.F., Kirsch, I.R., Bender, T.P. and Segal, S. (1986) Expression of a transfected c-*myc* oncogene inhibits differentiation of a mouse murine erythroleukaemia cell line. *Nature* **322**, 748-750.

Donti, E., Lanfrancone, L., Pellici, P.G., Birnie, G.D. and Dalla-Favera, R. (1991) Loss of amplification and appearance of a novel translocation site of the c-*myc* oncogene in HL-60 leukemia cells. *Cancer Genet. Cytogenet.* **56**, 57-64.

Dosaka-Akita, H., Rosenberg, J.D., Minna, J.D. and Birrer, M.J. (1991) A complex pattern of translational initiation and phosphorylation in L-Myc proteins. *Oncogene* **6**, 371-378.

Dunn, B.K., Cogliati, T., Cultraro, C.M., Bar-Ner, M. and Segal, S. (1994) Regulation of murine *max* (*myn*) parallels the regulation of c-*myc* in differentiating murine erythroleukemia cells. *Cell Growth Differ.* **5**, 847-854.

Dyson, N., Buchkovich, K., Whyte, P. and Harlow, E. (1989b) The cellular p107K protein that binds to the adenovirus E1A also associates with the large T antigens of SV40 and JC virus. *Cell* **58**, 249-255.

Dyson, N., Howley, P.M., Munger, K. and Harlow, E. (1989a) The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243**, 934-937.

Eagle, L.R., Yin, X., Brothman, A.R., Williams, B.J., Atkin, N.B. and Prochownik, E.V. (1995) Mutation of the *mxi1* gene in prostate cancer. *Nature Genet.* **9**, 249-255.

Eickholt, M., Nurnberg, W., Schadendorf, D., Welker, P., Grabbe, J. and Czarnetzki, B.M. (1993) Expression of c-*jun*, c-*myc* and c-*fos* proto-oncogenes regulated differently during mast-cell differentiation of U937 cells and peripheral blood monocytes. *J. Invest. Dermatol.* **100**, 480.

Eilers, M., Schrim, S., and Bishop, J.M. (1991) The MYC protein activates transcription of the α-*prothymosin* gene. *EMBO J.* **10**, 133-141.

Einat, M., Resnitsky, D. and Kimchi, A. (1985) Close link between reduction in c-Myc expression by interferon and $G_0/G_1$ arrest. *Nature* **313**, 597-600.

Eisenman, R.N., Tachibana, C.Y., Abrams, H. and Hann S.R. (1985) v-*myc* and c-*myc*-encoded proteins are associated with the nuclear matrix. *Mol. Cell. Biol.* **5**, 114-126.

Epstein, M.A. and Achong, B.G. (1983) The relationship of the EBV virus to Burkitt's lymphoma. In: The Epstein-Barr virus, M.A. Epstein and B.G. Achong, eds. (Berlin: Springer-Verlag), pp. 322-327.

Erickson, J.M., Rushford, C.L., Dorney, D.J., Wilson, G.N. and Schmickel, R.D. (1981) Structure and variation of human ribosomal DNA: Molecular analysis of cloned fragments. *Gene* **16**, 1-9.

Erisman, M.D., Rothberg, P.G., Diehl, R.E., Morse, C.C., Spandorfer, J.M. and Astrin, S.M. (1985) Deregulation of c-*myc* gene expression in human colon carcinoma is not accompanied by amplification or rearrangement of the gene. *Mol. Cell. Biol.* **5**, 1969-1976.

Eschenfeldt, W.H. and Berger, S.L. (1986) The human prothymosin α gene is polymorphic and induced upon growth stimulation: Evidence using a cloned cDNA. *Proc. Natl. Acad. Sci. USA* **83**, 9403-9407.

Evan, G.I. and Littlewood, T.D. (1993) The role of c-*myc* in cell growth. *Curr. Opin. Genet. Dev.* **3**, 44-49.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z. and Hancock, D.C. (1992) Induction of apoptosis in fibroblasts by c-Myc protein. *Cell* **69**, 119-128.

Evans, F.J. Hassan, N.M. (1993) Activation of the isotypes of protein kinase C and other kinases by phorbol esters of different biological activities. *ACS Symp. Series* **534**, 294-305.

Field, J.K. and Spandidos, D.A. (1990) The role of *ras* and *myc* oncogenes in human solid tumours and their relevence in diagnosis and prognosis. (Review) *Anticancer Res.* **10**, 1-22.

Finkelstein, R. and Weinberg, R. (1988) Differential regulation of N-*myc* and c-*myc* expression in F9 teratocarcinoma cells. *Oncogene Res.* **3**, 287-292

Freeman, R and King, B. (1972) Technique for the performance of the nitroblue tetrazolium (NBT) test. *J. Clin. Pathol.* **25**, 912.

Freytag, S.O. (1988) Enforced expression of the c-*myc* oncogene inhibits cell differentiation by precluding entry into a distinct predifferentiation state in $G_0/G_1$. *Mol. Cell. Biol.* **8**, 1614-1624.

Freytag, S.O., Dang, C.V. and Lee, W.M.F. (1990) Definition of the activities and properties of c-*myc* required to inhibit cell differentiation. *Cell Growth Differ.* **1**, 339-343.

Freytag, S.O. and Geddes, T.J. (1992) Reciprocal regulation of adipogenesis by c-Myc and C/EBPα. *Science* **256**, 379-382.

Gallagher, R.E., Collins, S., Trujillo, J., McCredie, K., Aheam, M., Tsai, S., Metzar, R., Aulahh, G., Ting, R., Ruscetti, F. and Gallo, R.C. (1979) Characterisation of the continuous differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. *Blood* **54**, 713-733.

Gaubatz, S., Meichle, A. and Eilers, M. (1994) An E-box element localized in the first intron mediates the regulation of the prothymosin α gene by c-*myc*. *Mol. Cell. Biol.* **14**, 3853-3862.

Gissmann, L. (1992) Human papillomaviruses and genital cancer. *Semin. Cancer Biol.* **3**, 253-261.

Goff, S.P., Gilboa, E., Witte, O.N. and Baltimore, D. (1980) Structure of the Abelson Murine Leukemia Virus genome and the homologous cellular gene: Studies with cloned viral DNA. *Cell* **22**, 777-785.

Gomez-Marquez, J., Segade, F., Dosil, M., Bustelo, X.R. and Freire, M. (1989) The expression of *prothymosin* α gene in T lymphocytes and leukaemic lymphoid cells is tied to lymphocyte proliferation. *J. Biol. Chem.* **264**, 8451-8454.

Gonda, T.J. and Metcalf, D. (1984) Expression of *myb*, *myc* and *fos* proto-oncogenes during the differentiation of a murine myeloid leukaemia. *Nature* **310**, 249-251.

Graham, S.V. and Birnie, G.D. (1988) Concomitant transcriptional and post-transcriptional control of messenger-RNA abundance during human myeloid cell differentiation. *Nucleic Acids Res.* **16**, 2523-2535.

Graham, S.V., Tindle, R.W. and Birnie, G.D. (1985) Variation in *myc* gene amplification and expression in sublines of HL60 cells. *Leukemia Res.* **9**, 239-247.

Gregor, P.D., Sawadogo, M. and Roeder R.G. (1990) The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. *Genes Dev.* **4**, 1730-1740.

Gregory, C.D., Dive, C., Henderson, S., Smith, C.A., Williams, G.T., Gordon, J. and Rickinson, A.B. (1991) Activation of Epstein-Barr virus latent genes protects human B cells from death from apoptosis. *Nature* **349**, 612-614.

Griep, A.E. and DeLuca, H.F. (1986) Decreased c-*myc* expression is an early event in retinoic acid-induced differentiation of F9 teratocarcinoma cells. *Proc. Natl. Acad. Sci.* USA **83**, 5539-5543.

Griep, A.E. and Westphal, H. (1988) Antisense *Myc* sequences induce differentiation of F9 cells. *Proc. Natl. Acad. Sci.* USA 85, 6806-6810.

Gu, W., Bhatia, K., Magrath, I.T., Dang, C.V. and Dalla-Favera, R. (1994) Binding and suppression of the Myc transactivation domain by p107. *Science* **264**, 251-254.

Hamilton, J.A., Veis, N., Bordun, A.M., Vairo, G., Gonda, T.J. and Phillips, W.A. (1989) Activation and proliferation signals in murine macrophages: relationships among c-*fos* and c-*myc* expression, phosphoinositide hydrolysis, superoxide formation and DNA synthesis. *J. Cell Physiol.* **141**, 618-626.

Hann, S.R. and Eisenman, R.N. (1984) Proteins encoded by the human c-*myc* oncogene: Differential expression in neoplastic cells. *Mol. Cell. Biol.* **4**, 2486-2497.

Hann, S.R., Dixit, M., Sears, R.C. and Sealy, L. (1994) The alternatively initiated c-Myc proteins differentially regulate transcription through a noncanonical DNA-binding site. *Genes Dev.* **8**, 2441-2452.

Hann, S.R., King, M.W., Bentley, D.L., Anderson, C.W. and Eisenman R.N. (1988) A non-AUG translation initiation in c-*myc* exon 1 generates an N-terminally distinct protein whose synthesis is disrupted in Burkitt's lymphoma. *Cell* **34**, 185-195.

Hann, S.R., Sloan-Brown, K. and Spoots, G.D. (1992) Translational activation of the non-AUG-initiated c-Myc 1 protein at high cell densities due to methionine deprivation. *Genes Dev.* **6**, 1229-1240.

Harrington, E.A., Bennett, M.R., Fanadi, A. and Evan, G.I. (1994) c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO J.* **13**, 3286-3295.

Harrington, E.A., Fanidi, A. and Evan, G.I. (1994b) Oncogenes and cell death. *Curr. Opin. Genet. Dev.* **4**, 120-129.

Harris, A.W., Pinkert, C., Crawford, M., Langdon, W., Brinster, R. and Adams, J.M. (1988) The Eμ-*myc* transgenic mouse: a model for high incidence of spontaneous lymphoma and leukemia of early B cells. *J. Exp. Med.* **167**, 353-371.

Harris, P. and Ralph, P. (1985) Human leukemic models of myelomonocytic development: A review of the HL-60 and U937 cell lines. *J. Leukocyte Biol.* **37**, 407-422.

Haupt, Y., Alexander, W.S., Barri, G., Klinken, S.P. and Adams, J.M. (1991) Novel zinc finger gene implicated as *myc* collaborator by retrovirally accelerated lymphomagenesis in Eμ-*myc* transgenic mice. *Cell* **65**, 753-763.

Hay, N., Bishop, J.M. and Levens, D. (1987) Regulatory elements that modulate expression of human c-*myc*. *Genes Dev* **1**, 659-671.

Hayashi, S.-I. and Murakami Y. (1995) Rapid and regulated degradation of ornithine decarboxylase. *Biochem. J.* **306**, 1-10.

Heikkila, R., Schwab, G., Wickstrom, E., Loke, S.L., Pluznik, D.H., Watt, R. and Neckers, L.M. (1987) A c-*myc* antisense oligonucleotide inhibits entry into S phase but not progression from G0 to G1. *Nature* **328**,445-449.

Henderson, S., Rowe, M., Gregory, C., Croom-Carter, D., Wang, F., Longnecker, R., Kieff, E. and Rickinson, A. (1991) Induction of *bcl*-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. *Cell* **65**, 1107-1115.

Hermeking, H. and Eick, D. (1994) Mediation of c-Myc-induced apoptosis by p53. *Science* **265**, 2091-2093.

Hickok, N.J., Wahlfors, J., Crozat, A., Halmekyto, H.M., Alhonen, L., Janne, J. and Janne, O.A. (1990) Human ornithine decarboxylase-encoding loci: nucleotide sequence of the expressed gene and characteristics of a pseudogene. *Gene* **93**, 257-263.

Hiebert, S.W., Lipp, M. and Nevins, J.R. (1989) E1A-dependent transactivation of the human *myc* promoter is mediated by the E2F factor. *Proc. Natl. Acad. Sci USA.* **86**, 3594-3598.

Hiebert, S.W., Packham, G., Strom, D.K., Haffner, R., Oren, M., Zambetti, G. and Cleveland, J.L. (1995) E2F-1/DP-1 induces p53 and overrides survival factors to trigger apoptosis. *Mol. Cell. Biol.* **15**, 6864-6874.

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D. and Korsmeyer, S.J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* **348**, 334-336.

Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C. (1991) *p53* mutations in human cancers. *Science* **253**, 49-53.

Holt, J.T., Redner, R.L. and Nienhuis, A.W. (1988) An oligomer complimentary to c-*myc* mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation. *Mol. Cell. Biol.* **8**, 963-973.

Hopewell, R. and Ziff, E.B. (1995) The nerve growth factor-responsive PC12 cell line does not express the Myc dimerization partner Max. *Mol. Cell. Biol.* **15**, 3470-3478.

Hu, Y.-F., Luescher, B., Admon, N., Mermod, N. and Tjian, R. (1990) Transcription factor AP4 contains multiple dimerization domains that regulate dimer specificity. *Genes Dev.* **4**, 1741-1752.

Human Gene Mapping II: (1991) *Cytogenet. Cell Genet.* **58**, parts 1-4.

Hurlin, P.J., Queva, C., Koskinen, P.J., Steingrimsson, E., Ayer, D.A., Copeland, N.G., Jenkins, N.A. and Eisenman, R.N. (1995) Mad3 and Mad4- Novel Max-interacting transcriptional repressors that suppress c-Myc dependent transformation and are expressed during neural and epidermal differentiation. *EMBO J.* **14**, 5646-5659.

Ingvarsson, S. (1990) The *myc* gene family proteins and their role in transformation and differentiation. *Semin. Cancer Biol.* **1**, 359-369.

Johnson, D.G., Schwarz, J.K., Cress, W.D. and Nevins, J.R. (1993) Expression of transcription factor E2F induces quiescent cells to enter S phase. *Nature* **365**, 349-352.

Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T. and Hishizuki, Y. (1983) Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. *J. Biol. Chem.* **258**, 6701-6704.

Kaddurah-Daouk, R., Greene, J.M., Baldwin, A.S. and Kingston R.E. (1987) Activation and repression of mammalian gene expression by the c-Myc protein. *Genes Dev.* **1**, 347-357.

Kato, G.J., Barrett, J., Villa-Garcia, M. and Dang, C.V. (1990) An amino terminal c-Myc domain required for neoplastic transformation activates transcription. *Mol. Cell. Biol.* **10**, 5914-5920.

Kato, G.J., Lee, W.M.F., Chen, L. and Dang, C.V. (1992) Max- functional domains and interaction with c-Myc. *Genes Dev* **6**, 81-92.

Kelly, K. and Siebenlist, U. (1986) The regulation and expression of c-*myc* in normal and malignant cells. *Ann. Rev. Immunol.* **4**, 317-338.

Kovesdi, I., Reichel, R. and Nevins, J.R. (1986) Identification of a cellular transcription factor involved in E1A transactivation. *Cell* **45**, 219-228.

Kretzner, L., Blackwood, E.M. and Eisenman, R.N. (1992) Myc and Max proteins possess distinct transcriptional activities. *Nature* **359**, 426-429.

Krumm, A., Meulia, T., Brunvand, M. and Groudine, M. (1992) The block to transcriptional elongation within the human c-*myc* gene is determined in the promoter-proximal region. *Genes Dev* **6**, 2201-2213.

Kurzrock, R., Gutterman, J.U. and Talpaz, M. (1988) The molecular genetics of Philadelphia-chromosome positive leukemias. *N. Engl. J. Med.* **319**, 990-998.

La Rocca, S.A., Crouch, D.H. and Gillespie, D.A.F. (1994) c-Myc inhibits myogenic differentiation and *myoD* expression by a mechanism which can be dissociated from cell transformation. *Oncogene* **9**, 3499-3508.

Lachman, H.M. and Skoultchi, A.I. (1984) Expression of c-*myc* changes during differentiation of mouse erythroleukaemia cells. *Nature* **310**, 592-594.

Lacy, J., Summers, W.P. and Summers, W.C. (1989) Post-transcriptional mechanisms of deregulation of *myc* following coversion of a human B-cell line by Epstein-Barr virus. *EMBO J.* **8**, 1973-1980.

Lagasse, E. and Clerc, R.G. (1988) Cloning and expression of 2 human genes encoding calcium-binding proteins that are regulated during myeloid differentiation. *Mol. Cell. Biol.* **8**, 2402-2410.

Lahoz, E.G, Xu, L., Schreiber-Agus, N. and DePinho, R.A. (1994) Suppression of Myc, but not E1a, transformation activity by Max-associated proteins Mad and Mxi1. *Proc. Natl. Acad. Sci. USA* **91**, 5503-5507.

Laird, P.W., van der Lugt, N.M.T., Clarke, A., Domen, J., Linders, K., McWhir, J., Berns, A. and Hooper, M.L. (1993) *In vivo* analysis of *Pim*-1 deficiency. *Nucleic Acids Res.* **21**, 4750-4755.

Lammie, G.A. and Peters, G. (1991) Chromosome 11q13 abnormalities in human cancer. *Cancer Cells* **3**, 413-420.

Land, H., Chen, A.C., Morgenstern, J.P., Parada, L.F. and Weinberg, R.A. (1986) Behavior of *myc* and *ras* oncogenes in transformation of rat embryo fibroblasts. *Mol. Cell. Biol.* **6**, 1917-1925.

Land, H. , Parada, L. and Weinberg, R.A. (1983a) Tumorigenic conversion of primary rat embryo fibroblasts requires at least two co-operating oncogenes. *Nature* **304**, 596-602.

Land, H., Parada, L.F. and Weinberg, R.A. (1983b) Cellular oncogenes and multistep carcinogenesis. *Science* **222**, 771-778.

Landschulz, W.H., Johnson, P.F. and McKnight, S.L. (1988) The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. *Science* **240**, 1759-1764.

Langdon, W.Y., Harris, A.W., Cory, S. and Adams, J.M. (1986) The c-*myc* oncogene perturbs B lymphocyte development in Eμ-*myc* transgenic mice. Cell 47, 11-18.

Larsson, L.-G., Ivhed, I., Gidlund, M., Pettersson, U., Vennstrom, B. and Nilsson, K. (1988) Phorbol ester-induced terminal differentiation is inhibited in human U937 monoblastic cells expressing a v-*myc* oncogene. *Proc. Natl. Acad. Sci.* USA **85**, 2638-2642.

Larsson, L.-G., Pettersson, M., Oberg, F., Nilsson, K. and Luscher, B. (1994) Expression of *mad*, *mxi1*, *max* and c-*myc* during induced differentiation of hematopoietic cells: opposite regulation of *mad* and c-*myc*. *Oncogene* **9**, 1247-1252.

Laskey, R.A. (1984) Radioisotope detection by fluorography and intensifying screens. In: Review booklet 23, Amersham Corporation, 2636 South Clearbook Drive, Arlington Heights, IL 60005.

Leder, A., Pattengale, P.K., Kuo, A., Stewart, T.A. and Leder, P. (1986) Consequences of widespread deregulation of the c-*myc* gene in transgenic mice: Multiple neoplasms and normal development. *Cell* **45**, 485-495.

Li, L.-h., Nerlov, C., Prendergast, G., MacGregor, D. and Ziff, E.B. (1994) c-Myc represses transcription in vivo by a novel mechanism dependent on the initiator element and Myc box II. *EMBO J.* **13**, 4070-4079.

Lipp, M., Schilling, R. and Bernhardt, G. (1989) Transactivation of human *myc*: The 2nd promoter is target for the stimulation by adenovirus E1A proteins. *Oncogene* **4**, 535-541.

Littlewood, T.D., Amati, B., Land, H. and Evan, G.I. (1992) Max and c-Myc:Max DNA-binding activities in cell-extracts. *Oncogene* 7, 1783-1792.

Lombardi, L., Newcomb, E.W. and Dalla-Favera, R. (1987) Pathogenesis of Burkitt lymphoma: Expression of an activated oncogene causes the tumorigenic conversion of EBV-infected human B lymphoblasts. *Cell* **49**, 161-170.

Lovec, H., Grzeschiczek, A., Kowalski, M.B. and Moroy, T. (1994) *Cyclin* D1 *bcl*-1 co-operates with *myc* genes in the generation of B-cell lymphoma in transgenic mice. *EMBO J.* **13**, 3487-3495.

Luk, G.D., Civin, C.I., Weissman, R.M. and Baylin, S.B. (1982) Ornithine decarboxylase: Essential for proliferation but not differentiation of human promyelocytic leukaemia cells. *Science* **216**, 75-77.

Luscher, B. and Eisenman, R.N. (1990) New light on Myc and Myb. Part 1. Myc. *Genes Dev.* **4**, 2025-2035.

Magrath, I. (1990) The pathogenesis of Birkitt's lymphoma. *Adv. Cancer Res.* **55**, 133-270.

Maheswaran, S., Lee, H. and Sonenshein, G.E. (1994) Intracellular association of the protein product of the c-*myc* oncogene with the TATA-binding protein. *Mol.Cell. Biol.* **14**, 1147-1152.

Marcu, K.B., Bossone, S.A. and Patel, A.J. (1992) Myc function and regulation. *Annu. Rev. Biochem.* **61**, 809-860.

Maruyama, K., Schiavi, S.C., Huse, W., Johnson, G.L. and Ruley, H.E. (1987) Myc and E1A oncogenes alter the responsiveness of PC12 cells to nerve growth factor and block differentiation. *Oncogene* **1**, 361-367.

McCachren, S.S., Nichols, J., Kaufman, R.E. and Niedel, J.E. (1986) Dibutyryl cyclic adenosine monophosphate reduces expression of c-*myc* during HL60 differentiation. *Blood* **68**, 412-416.

McCarthy, D., San Miguel, J., Freake, H., Green, P., Zola, H., Catovsky, D. and Goldmann, J. (1983) 1,25 Dihydroxyvitamin $D_3$ inhibits proliferation of human promyelocytic leukemia (HL-60) cells and induces monocyte-macrophage differentiation in HL-60 and normal human bone marrow cells. *Leukemia. Res.* 7, 51-55.

McDonnell, T.J., Deane, N., Platt, F.M., Numez, G., Jaeger, V., Mckearn, J.P. and Korsmeyer, S.J. (1989) *bcl*-2 immunoglobulin transgenic mice demonstrate extended B-cell survival and follicular lymphoproliferation. *Cell* **57**, 79-88.

McDonnell, T.J. and Korsmeyer, S.J. (1991) Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14;18). *Nature* **349**, 254-256.

Miner, J.H. and Wold, B.J. (1991) c-*myc* inhibition of MyoD and myogenein - initiated myogenic differentiation. *Mol. Cell. Biol.* **11**, 2842-2851.

Mitchell, L.S., Neill, R.A. and Birnie, G.D. (1992) Temporal relationships between induced changes in c-*myc* mRNA abundance, proliferation and differentiation in HL60 cells. *Differentiation* **49**, 119-125.

Mitchell, R.L., Henning-Chubb, C., Huberman, E. and Verma, I.M. (1986) c-*fos* expression in neither sufficient nor obligatory for differentiation of monomyelocytes to macrophages. *Cell* **45**, 497-504.

Mitchell, R.L., Zokas, L., Schreiber, R.D. and Verma, I.M. (1985) Rapid induction of the expression of the proto-oncogene *fos* during human monocytic differentiation. *Cell* **40**, 209-217.

Muller, R., Curran, T., Muller, D. and Guilbert, L. (1985) Induction of c-*fos* during myelomonocytic differentiation and macrophage proliferation. *Nature* **314**, 546-548.

Muller, R., Muller, D. and Guilbert, L. (1984) Differential expression of c-*fos* in hematopoietic cells: correlation with differentiation of myelomonocytic cells in vitro. *EMBO J.* **3**, 1887-1890.

Murray, M.J., Cunningham, J.M., Parada, L.F., Dautry, F., Lebowitz, P. and Weinberg, R.A. (1983) *Onc* gene amplification in promyelocytic leukemia cell line HL-60 and primary leukemic cells of the same patient. *Cell* **33**, 749-757.

Murre, C., McCae, P.S., Vaessin, H., Caudy, M., Jan, Y.N., Cabrera, C.V., Buskin, J.N., Hauseka, S.D., Lassar, A.B., Weintraub, H. and Baltimore, D. (1989) Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58**, 537-544.

Nakagoshi, H., Kaeiishii, C., Sawazaki, T., Mizuguchi, G. and Ishii, S. (1992) Transcriptional activation of the c-*myc* gene by the c-*myb* and B-*myc* gene products. *Oncogene* **7**, 1233-1240.

Nakiki, T., Mita, S., Yamamoto, S., Nakadate, T. and Kato, R. (1984) Inhibition by palmitoylcarnitine of adhesion and morphological changes in HL60 cells induced by 12-o-tetradecanoylphorbol-13-acetate. *Cancer Res.* **44**, 1908-1912.

Nau, N.M., Brooks, B.J., Battey, J., Sausville, E., Gazdar, A.F., Kirsch, I.R., McBride, O.W., Bertness, V., Hollis, G.F. and Minna J.D. (1985) L-*myc*, a new *myc*-related gene amplified and expressed in human small cell lung cancer. *Nature* **318**, 69-73.

Needleman, S.W., Kraus, M.H., Srivastava, S.K., Levine, P.H. and Aaronson, S.A. (1986) High frequency of N-*ras* activation in acute myelogenous leukemia. *Blood* **67**, 753-757.

Neil, J.C., Forrest, D., Doggett, D.L. and Mullins, J.I. (1987) The role of feline leukaemia virus in naturally occuring leukaemias. *Cancer Surv.* **6**, 117-137.

Newbold, R.F. and Overell, R.W. (1983) Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-*ras* oncogene. *Nature* **304**, 648-651.

Novell, P.C. and Croce, C.M. (1987) Cytogenetics of neoplasia. In: Development and recognition of the transformed cell. M.I. Greene and T. Hamaoka, editors. Plenum Publishing Corporation. New York.

Packham, G. and Cleveland, J.L. (1994) Ornithine decarboxylase is a mediator of c-Myc-induced apoptosis. *Mol. Cell. Biol.* **14**, 5741-5747.

Payne, G.S., Bishop, J.M. and Varmus, H.E. 1982 Multiple arrangements of viral-DNA and an activated host oncogene in bursal lymphomas. *Nature* **295**, 209-214.

Pegg, A.E. (1986) Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem. J.* **234**, 249-262.

Pellici, S., Knowles, D.M., Magrath, I. and Dalla-Favera, R. (1986) Chromosomal breakpoints and structural alterations of the c-*myc* locus differ in endemic and sporadic forms of Burkitt lymphoma. *Proc. Natl. Acad. Sci.* USA **83**, 2984-2988.

Penn L.J.Z., Laufer, E.M. and Land, H. (1990) C-MYC: evidence for multiple regulatory functions. *Semin. Cancer Biol.* **1**, 69-80.

Peters, G. (1990) Oncogenes at viral integration sites. *Cell Growth Differ.* **1**,503-510.

Pfister, H. (1992) Human papillomaviruses and skin cancer. *Semin. Cancer Biol.* **3**, 263-271.

Phillip, A., Schneider, A., Vasrik, I., Finke, K., Xiong, Y., Beach, D., Alitalo, K. and Eilers, M. (1994) Repression of cyclin D1: a novel function of MYC. *Mol. Cell. Biol.* **14**, 4032-4043.

Pohjanpelto, P., Hollta, E. and Janne,O.A. (1985)Mutant strains of chinese hamster ovary cells with no detectable ornithine decarboxylase activity. *Mol. Cell. Biol.* **5**,1385-1390.

Postel, E.H., Berberich, S.J., Flint, S.J. and Ferrone, C.A. (1993) Human c-*myc* transcription factor PuF identified as NM23-H2 nucleotide diphosphate kinase. *Science* **261**, 478-480.

Postel, E.H., Mango, S.E. and Flint, S.J. (1989) A nuclease-hypersensitive element of the human c-*myc* promoter interacts with a transcription initiation factor. *Mol. Cell. Biol.* **9**, 5123-5133.

Prendergast, G.C., Lawe, D. and Ziff, E.B. (1991) Association of Myn, the murine homolog of Max with c-Myc stimulates methylation-sensitive DNA binding and Ras cotransformation. *Cell* **65**, 395-407.

Principato, M.A., Cleveland, J.L., Rapp, U.R., Holmes, K.L, Pierce, J.H., Morse III, H.C. and Klinken, S.P. (1990) 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**, 3562-3568.

Prochownik, E.V. and Kukowska, J. (1986) Deregulated expression of c-*myc* by murine erythroleukaemia cells prevents differentiation. *Nature* **322**, 848-850.

Rabbitts, P.H., Watson, J.V., Lamond, A., Forster, A., Stinson, M.A., Evan, G., Fischer, W., Atherton, E., Sheppard, R. and Rabbitts, T.H. (1985) Metabolism of c-*myc* gene products: c-*myc* messenger RNA and protein expression in the cell cycle. *EMBO J.* **4**, 2009-2015.

Rabbitts, T.H., Forster, A, Hamlyn, P.H. and Baer, R. (1984) Effect of somatic mutations within translocated c-*myc* genes in Burkitt's lymphoma. *Nature* **309**, 592-597.

Rabbitts, T.H., Hamlyn, P.H. and Baer, R. (1983) Altered nucleotide sequences of a translocated c-*myc* in Burkitt lymphoma. *Nature* **306**, 760-765.

Raffeld, M. and Jaffe, E.S. (1991) bcl-1, t(11;14), and mantle cell-derived lymphomas. *Blood* **78**, 259-263.

Ramsay, G., Stanton, L., Schwab, M. and Bishop, J.M. (1986) The human proto-oncogene N-*myc* encodes nuclear proteins that bind DNA. *Mol. Cell. Biol.* **6**, 4450-4457.

Ray, D. and Robert-Lesenges, J. (1989) Coexistance of a c-*myc* messenger RNA initiated in intron 1 with the normal c-*myc* messenger RNA and similar regulation of both transcripts in mammalian cells. *Oncogene Res.* **5**, 73-78.

Raynaud, S.D., Bekri, S., Leroux, D., Grosgeorge, J., Klein, B., Bastard, C., Gaudray, P. and Simon, M-P. (1993) Expanded range of 11q13 breakpoints with differing patterns of *cyclin* D1 expression in B-cell malignancies. *Genes Chrom. Cancer* **8**, 80-87.

Reed, J.C. (1994) Bcl-2 and the regulation of programmed cell death. *J. Cell Biol.* **124**, 1-6.

Reid, A.J. (1992) The expression of the stem cell inhibitor/macrophage inflammatory protein-1α and macrophage inflammatory protein-1β mRNA in murine macrophages. Ph.D. Thesis, University of Glasgow.

Reitsma, P., Rothberg, P., Astrin, S., Trial, J., Bar-Shavit, Z., Hall, S., Teitelbaum, S. and Kahn, A. (1983) Regulation of *myc* gene expression in HL-60 leukemia cells by a vitamin D metabolite. *Nature* **306**, 492-494.

Resar, L.M.S., Dolde, C., Barrett, J.F. and Dang, C.V. (1993) B-*myc* inhibits neoplastic transformation and transcriptional activation by c-*myc*. *Mol. Cell. Biol.* **13**, 1130-1136.

Riggs, K.J., Merrell, K.T., Wilson, G. and Calame, K. (1991) Common factor 1 is a transcriptional activator which binds in the c-*myc* promoter, the skeletal α-actin promoter and the immunoglobulin heavy-chain enhancer. *Mol. Cell. Biol.* **11**, 1765-1769.

Rogers, R.P., Strominger, J.L. and Speck, S.H. (1992) Epstein-Barr virus in B lymphocytes-viral gene expression and function in latency. *Adv. Cancer Res.* **58**, 1-26.

Rosenbaum, H., Harris, A.W., Bath, M.L., McNeall, J., Webb, E., Adams, J.M. and Cory, S. (1990) An Eμ-v-*abl* transgene elicits plasmacytomas in concert with an activated *myc* gene. *EMBO J.* **9**, 897-905.

Rosenberg, C.L., Kim, H.G., Shows, T.B., Kronenberg, H.M. and Arnold, A. (1991) Rearrangement and over expression of D11S287E, a candidate oncogene on chromosome 11q13 in benign parathyroid tumors. *Oncogene* **6**, 449-453.

Rothberg, P.G., Erisman, M.D., Diehl, R.E., Rovigatti, U.G. and Astrin, S.M. (1984) Structure and expression of the oncogene c-*myc* in fresh tumour material from patients with haemopoietic malignancies. *Mol. Cell. Biol.* **4**, 1096-1103.

Rowe, M., Rowe, D.T., Gregory, C.D., Young, L.S., Farrell, P.J., Rupani, H. and Rickinson, A.B. (1987) Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. *EMBO J.* **6**, 2743-2751.

Roy, A.L., Carruthers, C., Gutjahr, T. and Roeder, R.G. (1993) Direct role for Myc in transcriptional initiation mediated by interactions with TFII-I. *Nature* **365**, 356-361.

Rius, C. and Aller, P. (1989) Modulation of ornithine decarboxylase gene transcript levels by differentiation inducers in human promyelocytic leukemia HL60 cells. *Cell Differ. Dev.* **28**, 39-46.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning, a laboratory manual: 2nd ed., Cold Spring Harbor Laboratory Press.

Samuelsson, L., Stromberg, K., Vikman, K., Bjursell, G. and Enerback, S. (1991) The CCAAT/enhancer binding protein and its role in adipocyte differentiation: evidence for direct involvement in terminal adipocyte development. *EMBO J.* **10**, 3787-3793.

Sarnow, P., Ho, Y.S. Williams, J. and Levine, A.J. (1982) Adenovirus E1b-58 kDa tumour antigen and SV40 large tumour antigen are physically associated with the same 54 kDa cellular protein in transformed cells. *Cell* **28**, 387-394.

Sawyers, C.L., Denny, C.T. and Witte, O.N. (1991) Leukaemia and the disruption of normal hematopoiesis. *Cell* **64**, 337-350.

Schmidt, E.V., Pattengale, P.K., Weir, L. and Leder, P. (1988) Transgenic mice bearing the human c-*myc* gene activated by an immunoglobulin enhancer: a pre-B lymphoma model. *Proc. Natl. Acad. Sci.* USA **85**, 6047-6051.

Schreiber-Agus, N., Chin, L., Chen, K., Torres, R., Rao, G., Guida, P., Skoultchi, A.I. and DePinho, R.A. (1995) An amino-terminal domain of Mxi1 mediates anti-Myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor Sin3. *Cell* **80**, 777-786.

Schwab, M., Alitalo, K., Klempnauer, K-L., Varmus, H.E., Bishop, J.M., Gilbert, F., Brodeur, G., Goldstein, M. and Trent, J. (1983) Amplified DNA with limited homology to *myc* cellular oncogene is by human neuroblastoma cell lines and a neuroblatoma tumour. *Nature* **305**, 245-248.

Schwartz, R.C., Stanton, L.W., Riley, S.C., Marcu, K.B. and Witte, O.N. (1986) Synergism of v-*myc* and v-Ha-*ras* in the in vitro neoplastic progression of murine lymphoid cells. *Mol. Cell. Biol.* **6**, 3221-3231.

Scott, R., Florine, D., Wille, J. and Yun, K. (1982) Coupling of growth arrest and differentiation at a distinct state in the $G_0$ phase of the cell cycle. *Proc. Natl. Acad. Sci.* USA **80**, 2495-2499.

Selten, G., Cuypers, H.T. and Berns, A. (1985) Proviral activation of the putative oncogene *Pim*-1 in MuLV induced T-cell lymphomas. *EMBO J.* **4**, 1793-1798.

Serrano, M., Hannon, G.J. and Beach D. (1993) A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366**, 704-707.

Seto, E., Shi, Y and Shenk, T. (1991) YY-1 is an initiator sequence binding protein that directs and activates transcription *in vitro*. *Nature* **354**, 241-245.

Sharkey, N.A., Leach, K.L. and Blumberg, P.M. (1984) Competitive inhibition by diacylglycerol of specific phorbol ester binding. *Proc. Natl. Acad. Sci.* USA **81**, 607-610.

Shi, Y., Seto, E., Chang, L.S. and Shenk, T. (1991) Transcriptional repression by YY-1, a human GL1-Kruppel-related protein and releif of repression by adenovirus E1A protein. *Cell* **67**, 377-388.

Shirodkar, S., Ewen, M., DeCaprio, J.A., Morgan, J., Livingston, D.M., and Chittenden, T. (1992) The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-related manner. *Cell* **68**, 157-166.

Shrivastava, A., Saleque, S., Kalpana, G.V., Artandi, S., Goff, S.P. and Calame, K. (1993) Inhibition of transcriptional regulator Yin-Yang-1 by association with c-Myc. *Science* **262**, 1889-1892.

Siebenlist, U., Hennighausen, L., Battey, J. and Leder, P. (1984) Chromatin structure and protein-binding in the putative regulatory region of the c-*myc* gene in Burkitt-lymphoma. *Cell* **37**, 381-391.

Smale, S.T. and Baltimore, D. (1989) The "Initiator" as a transcription control element. *Cell* **57**, 103-113.

Smeyne, R., Vendrell, M., Hayward, M., Baker, S., Miao, G., Schilling, K., Robertson, L., Curran, T., and Morgan, J. (1993) Continuous c-*fos* expression precedes programmed cell death *in vivo*. *Nature* **363**, 166-169.

Solomon, D.L.C., Amati, B. and Land, H. (1993) Dinstinct DNA-binding preferences for the c-Myc:Max and Max:Max dimers. *Nucleic Acids Res.* **21**, 5572-5576.

Spencer, C.A. and Groudine M. (1991) Control of c-*myc* regulation in normal and malignant cells. *Adv. Cancer Res.* **56**, 1-48.

Stewart, T.A., Bellve, A.R. and Leder, P. (1984) Transcription and promoter usage of the *myc* gene in normal somatic and spermatogenic cells. *Science* **226**, 707-710.

Stone, J., de Lange, T., Ramsay, G., Jakobovits, E., Bishop, J.M., Varmus, H. and Lee, W. (1987) Definition of regions in human c-*myc* that are involved in transformation and nuclear localization. *Mol. Cell. Biol.* **7**, 1697-1709.

Strasser, A., Harris, A.W., Bath, M.L. amd Cory, S. (1990) Novel primitive lymphoid tumours induced in transgenic mice by co-operation between *myc* and *bcl*-2. *Nature* **348**,. 331-333.

Studzinski, G.P., Brelvi, Z.S., Feldman S.C. and Watt R.A. (1986) Participation of the c-Myc protein in DNA synthesis of human cells. *Science* **234**, 246-470.

Suggs, S.V., Wallace, R.B., Hirose, T., Kawashima, E.M. and Itakura, K. (1981) Use of synthetic oligonucleotides as hybridization probes. 3. isolation of cloned cDNA sequences from human *$\beta_2$-microglobulin*. *Proc. Natl. Acad. Sci.* USA 78, 6613-6617.

Sugiyama, A., Kume, A., Nemoto, K., Lee, S.Y., Asami, Y., Nemoto, F., Nishimura, S. and Kuchino, Y. (1989) Isolation and characterisation of S-*myc*, a member of the rat *myc* gene family. *Proc. Natl. Acad. Sci. USA* **86**, 9144-9148.

Tabor, C.W. and Tabor, H, (1984) Polyamines. *Annu. Rev. Biochem.* **53**, 749-790.

Tahara, H., Otani, S., Matsui-Yuasa, I., Koyama, H., Nishizawa, Y., Morisawa, S. and Morii, H. (1991) Role of putrescine in interleukin 1β production in human histiocytic lymphoma cell line U937. *J. Cell Physiol.* **147**, 199-207.

Taub, R., Kirsch, I., Morton, C., Lenoir, G.M., Swan, D., Tronick, S., Aaronson, S. and Leder, P. (1982) Translocation of the c-*myc* gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. *Proc. Natl. Acad. Sci.* USA **79**, 7837-7841.

Taub, R., Moulding, C., Battey, J., Latt, S. Lenoir, G.M. and Leder, P. (1984) Activation and somatic mutation of the translocated c-*myc* gene in Burkitt lymphoma cells. *Cell* **36**, 339-348.

Thalmeier, K., Synovik, H., Mertz, R., Winnacher, E-L and Lipp, M. (1989) Nuclear factor E2F mediates basic transcription and transactivation by E1A of the human *myc* promoter. *Genes Dev* **3**, 527-536.

Toksoz, D., Bunce, C.M., Stone, P.C.W., Michell, R.H. and Brown, G. (1982) Variant cell lines from the promyelocyte line HL60. *Leukaemia Res.* **6**, 491-498.

Tsiftsoglou, A.S. and Robinson, S.H. (1985) Differentiation of leukemic cell lines: A review focusing on murine erythroleukemia and human HL-60 cells. *Int. J. Cell Cloning* **3**, 349-366.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E. and Croce, C.M. (1985) The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science* **229**, 1390-1393.

van der Lugt, N.M.T., Domen, J., Verhoeven, E., Linders, K., van der Gulden, H., Allen, J. and Berns, A. (1995) Proviral tagging in Eμ-*myc* transgenic mice lacking the *pim*-1 proto-oncogene leads to compensatory activation of *pim*-2. *EMBO J.* **14**, 1536-2544.

van Lohuizen, M. and Berns, A. (1990) Tumorigenesis by slow-transforming retroviruses-an update. *Biochem. Biophys. Acta* **1032**, 213-315.

van Lohuizen, M., Verbeek, S., Scheijen, B., Wientjens, E., van der Gulden, H. and Berns, A. (1991) Identification of co-operating oncogenes in Eμ-*myc* transgenic mice by provirus tagging. *Cell* **65**, 737-752.

Vaux, D., Cory, S. and Adams, J. (1988) *Bcl*-2 gene promotes haemopoietic cell survival and co-operates with c-*myc* to immortalize pre-B cells. *Nature* **335**, 440-442.

Vennstrom, B., Sheiness, D., Zabielski, J. and Bishop, J.M. (1982) Isolation and Characterization of c-*myc*, a cellular homolog of the oncogene (v-*myc*) of avian myelocytomatosis virus strain 29. *J. Virol.* **42**, 773-779.

Verbeek, S., van Lohuizen, M., van der Valk, M., Domen, J., Kraal, G. and Berns, A. (1991) Mice bearing the Eμ-*myc* and Eμ-*pim*-1 transgenes develop pre-B-cell leukemia prenatally. *Mol. Cell. Biol.* **11**, 1176-1179.

Vogelstein, B. and Kinzler, K.W. (1993) The multistep nature of cancer. *Trends Genet.* **9**, 138-141.

Wagner, A.J., Lebeau, M.M., Diaz, M.O. and Hay, N. (1992) Expression, regulation and chromosomal localization of the *max* gene. *Proc. Natl. Acad. Sci.* USA **89**, 3111-3115.

Wagner, A.J., Meyers, C., Laimins, L.A. and Hay, N. (1993) c-Myc induces the expression and activity of ornithine decarboxylase. *Cell Growth and Differ.* **4**, 879-883.

Wagner, A.J., Small, M.B. and Hay, N. (1993) Myc-mediated apoptosis is blocked by ectopic expression of *bcl*-2. *Mol. Cell. Biol.* **13**, 2432-2440.

Wang, Z.Q., Ovitt, C., Grigoriadis, A.E., Mohlesteinlein, U., Ruther, U. and Wagnre, E.F. (1992) Bone and hematopoietic defects in mice lacking c-*fos*. *Nature* **360**, 741-745.

Watson, D.K., Psallidopoulos, M.C., Samuel, K.P., Dalla-Favera, R. and Papas, T. (1983) Nucleotide-sequence analysis of human c-*myc* locus, chicken homologue, and myelocytomatosis virus MC29 transforming gene reveals a highly conserved gene product. *Proc. Natl. Acad. Sci.* U.S.A. **80**, 3642-3645.

Ways, D.K., Dodd, R.C. and Earp, H.S. (1987) Dissimilar effects of phorbol ester and diacylglycerol derivative on protein kinase C activity in the monoblastoid U937 cell. *Cancer Res.* **47**, 3344-3350.

Webb, E., Barri, G., Cory, S. and Adams, J.M. (1989) Lymphomagenesis in Eμ-*myc* transgenic mice does not require transgene rearrangements or mutation of *myc* exon 1. *Mol. Cell. Biol.* **6**, 475-480.

Werness, B.A., Levine, A.J. and Howley, P.M. (1990) Association of the papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248**, 76-79.

Westin, E., Wong-Staal, F., Gelmann, E., Dalla-Favera, R., Papas, T., Lautenberger, J., Allessandra, E., Reddy, E., Tronick, S., Aaronson, S. and Gallo, R.C. (1982) Expression of cellular homologues of retroviral *onc* genes in human hematopoietic cells. *Proc. Natl. Acad. Sci.* USA 79, 2490-2494.

Wolf, D. and Rotter, V. (1985) Major deletions in the gene encoding the p53 tumour-antigen cause lack of p53 expression in HL-60 cells. *Proc. Natl. Acad. Sci.* USA **82**, 790-794.

Whyte, P., Buchkovich, K.J., Horwitz, J.M., Friend, S.H., Raybuck, M., Weinberg, R.A. and Harlow, E. (1988) Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature* **334**, 124-129.

Whyte, P., Williamson, N.M. and Harlow, E. (1989) Cellular targets for transformation by the adenovirus E1A protein. *Cell* **56**, 57-75.

Wickstrom, E.L., Bacon, T.A., Gonzalez, A., Freeman, D.L. Lyman, G.H. and Wickstrom, E. (1988) Human promyelocytic leukemia HL-60 cell proliferation and c-Myc protein expression are inhibited by an antisense pentadecadeoxynucleotide targeted against c-*myc* mRNA. *Proc. Natl. Acad. Sci* USA **85**, 1028-1032.

Yeh, W.-c. and McKnight, S.L. (1995) Regulation of adipose maturation and energy homeostasis. *Curr. Opin. Cell Biol.* 7, 885-890.

Yokota, J., Tsunetsugu-Yokota, Y., Battifora, H., Le Fevre, C. and Cline, M.J. (1986) Alterations in *myc*, *myb* and *ras*$^{Ha}$ proto-oncogenes in cancers are frequent and show clinical correlation. *Science* **231**, 261-265.

Zervos, A.S., Gyuris, J. and Brent, R. (1993) Mxi1, a protein that specifically interacts with Max to bind Myc:Max recognition sites. *Cell* **72**, 223-232.

Zimmerman, K. and Alt, F.W. (1990) Expression and function of *myc* family genes. *Crit. Rev. in Oncogenesis* **2**, 75-95.