Functional Analysis of a Naturally Occurring Mutant *myc* Gene

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The work in this thesis was carried out by myself, unless otherwise stated.

ABBREVIATIONS

ATP	adenosine triphosphate
bHLH	basic region, helix-loop-helix
bp	base pairs
cDNA	complementary DNA
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulphoxide
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetra-acetic acid
EMSA	electrophoretic mobility shift assay
FeLV	feline leukaemia virus
GST	glutathione S-transferase
IPTG	indolpropyl β-D thiogalactopyranoside
kb	kilobase
LZ	leucine zipper
MOPS	sodium morpholinopropane sulphonic acid
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PVDF	polyvinylidene fluoride
SDS	sodium dodecyl sulphate
TBS	Tris buffered saline
TEMED	N,N,N,N,-tetramethylethylenediamine
X-gal	5-bromo 4-chloro 3-indolyl β-galactoside

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Summary

The c-*myc* gene plays a role in the aetiology of various cancers of humans and animals. Feline leukaemia virus has also been demonstrated to have oncogenic potential, and in approximately one third of tumours where FeLV is present aberrant expression of a *myc* gene occurs. This changed expression pattern is mainly due to FeLV transduction of the *myc* gene, but can also occur by insertional mutagenesis.

Until recently v-*myc* genes have been found to be virtually equivalent to c-*myc*, with few mutations in the coding sequence. This project focuses on a FeLV-transduced v-*myc*, termed T17-*myc*, which is exceptional in that it is highly mutated. Mutations include partial loss of a domain previously identified as crucial for transformation, as well as an insertion in the basic region (BR) sequence-specific DNA binding domain.

The aim of this work was to characterise the mutant oncogene at the biological and biochemical level, to discover whether various Myc functions could be dissociated using the mutant.

I have shown that the original mutations are maintained in secondary lymphomas which occurred rapidly after inoculation of the T17 virus complex, arguing that the mutant is a relatively efficient oncogene. Despite its apparent *in vivo* efficiency, it was transformation defective in chick embryo fibroblasts, and was unable to induce apoptosis in the same cells. Chimaeric genes showed that the transformation and apoptosis defects were caused by the N-terminal mutation. However, the C-terminal BR mutation independently lowered transformation efficiency and growth rate, although the mutation did not prevent binding to DNA along with Max, either *in vitro* or *in vivo*.

Analysis of gene expression in the original T17 lymphoma-derived cell line showed that putative Myc regulated, and Myc regulating genes were expressed in the mutant Myc cell line, although the mutant Myc was able to interact with the transcriptional repressor p107 *in vitro*.

The data presented in this thesis are consistent with a model where mutations in the N-terminal domain of Myc abolish the negative growth effects of the *myc* gene, with relatively little consequence for its oncogenic function in T cells. Also consistent with these data is the ability of Myc to interact with cell type specific factors involved in transcription of Myc-regulated genes.

CHAPTER 1

1 Introduction

The *myc* gene was originally discovered as a transduced gene in avian myelocytomatosis virus MC29 (1-3). The gene comes from a family of cellular genes of which the best characterised are : c-*myc*, L-*myc*, and N-*myc*. The first to be discovered due to homology with v-*myc* (1) and the most intensively studied is c-*myc*, where "c" denotes cellular (4). L-*myc* was discovered in a small cell lung carcinoma (5), while N-*myc* was found as a c-*myc* related gene which was amplified in some neuroblastomas (6).

All three *myc* genes are composed of three exons. Exons two and three code for the major protein product, while exon one contains predominantly regulatory sequences necessary for the control of *myc* expression (7). There are two translational products of c-*myc*, the major 64kD form is initiated from an AUG codon in exon 2, while the minor 67kD protein is initiated from a CUG codon at the 3' end of exon 1 (8). Loss of the first exon has been shown to increase the oncogenic potential of all three *myc* homologues (9).

Both the *myc* RNA and protein products have short half lives. *myc* mRNA has been shown to have a half life of ~10 minutes (10), which could be dramatically extended by inhibition of protein synthesis in some cell lines, though not in others. This variability suggests that post transcriptional control of *myc* is mediated by different factors in different cell types (10). Both c-Myc translation products are phosphoproteins localised in the nucleus, with a short half life of ~25 minutes (11).

Since the original discovery of *myc*, the gene has been implicated in a variety of cancers in both humans and animals and has been found to be deregulated by diverse mechanisms, such as proviral insertion, viral transduction or chromosomal translocation (Table 1.1). The last mechanism was noted first in Burkitt's lymphoma, where the *myc* gene comes

Table 1.1 Examples of tumour-specific alterations of c-*myc* in different species.

Alteration to c-*myc*	Species	Control by	Tumour Type	Reference
Retroviral Transduction	Chicken	ALV, REV	Leukaemia, Lymphoma, Carcinoma	(12)
	Cat	FeLV	T cell lymphoma	(13)
Retroviral Insertion	Chicken	ALV, REV	B cell lymphoma	(14,15)
	Mouse	MuLV,MCF	T cell lymphoma	(16,17)
	Cat	FeLV	" "	(18)
	Rat	MuLV	" "	(19)
Other Insertion	Mouse	IAP	Plasmacytoma	(20)
	Dog	Retroposon	Transmissible venereal tumour	(21)
Chromosomal Translocation	Man	IgH, IgL	B cell lymphoma	(22,23)
		TCRα	T cell lymphoma	(24,25)
		t(3:8)	Renal cell carcinoma	(26)
	Mouse	IgH	Plasmacytoma	(23)
	Rat	IgH	Immunocytoma	(27)
Amplification	Man	DM, HSR	Lung carcinoma	(28)
		DM, HSR	Colon carcinoma	(29)
		DM	Gastric carcinoma	(30)
		DM, HSR	Myeloid leukaemia	(31)
		DM	Glioblastoma	(32)

Key: ALV, avian leukosis virus; REV, reticuloendotheliosis virus; FeLV, feline leukaemia virus; MuLV, murine leukaemia virus; MCF, mink cell focus-forming virus; IAP, Intracisternal A-Particle element; TCRα, T cell receptor α chain locus; IgH, immunoglobulin heavy chain locus; IgL, immunoglobulin κ or λ light chain locus; DM, double minute chromosomes; HSR, homogeneously staining region.

under the control of an immunoglobulin enhancer element (22,33). The most common translocation is t(8:14), where a reciprocal recombination occurs between the c-*myc* locus at band q24 on chromosome 8, and the immunoglobulin heavy chain locus at band q32 on chromosome 14 (23). Other than the translocation of c-*myc* to the immunoglobulin locus there is strong evidence that Epstein-Barr virus (EBV) plays some role in lymphomagenesis. In African children early EBV infection with a high multiplicity has been shown to correlate with lymphoma, indeed 97% of African Burkitt lymphoma cells have multiple copies of the EBV genome in them (33).

1.1 Biological Effects of c-*myc*

1.1.1 Tumours

Evidence that c-Myc is important for the normal function of cells comes from the association between the deregulation of c-*myc* and oncogenesis, with c-*myc* presenting a frequent target for activation in a variety of cancers (Table 1.1). From early work c-*myc* was suggested to act as an immortalising gene, in the same mould as E1a and large T antigen (7), since each of these gene products can partially transform primary cells in culture without inducing a complete tumourigenic phenotype (7). Indeed secondary genetic events are required for *in vitro* systems which measure the ability of Myc to induce transformation (34), while early *in vivo* work on promyelocytic leukaemia and Burkitt's lymphoma demonstrated the need for cooperating oncogenes in human cancer (35). Studies involving a variety of human cancers confirmed that the c-*myc* gene was expressed in all tumour types tested and in almost all individual tumours (36). This may have been taken simply as evidence of an essential requirement for c-*myc* in cell division, but this seemed unlikely from the observation that c-*myc* expression was aberrant in 71% of tumours compared to normal tissue from the same organ of each patient (36).

In a model system utilising exogenous c-*myc* expressed under the control of viral promoters in fibroblast cell lines, the fibroblasts were able to induce tumours when injected subcutaneously into nude mice, or syngeneic rats (37). In this experiment there

was no marked alteration in cell morphology or anchorage independent growth of the cell lines overexpressing c-*myc* (37).

Another approach to investigating the biological significance of *myc* has been the use of transgenic technology. Two methods have been developed which allow either the direct targeting of the genetic lesion to a specific cell type, or the widespread overexpression in a variety of tissues.

Using cell type specific enhancers the first of these methods has been used to target *myc* expression to various tissues including; breast, utilising a mouse mammary tumour virus LTR (38); lymphoid, targeted using immunoglobulin heavy chain enhancer (Eμ-*myc*) (39), as well as several other tissue types, e.g. liver, pancreas, heart, and lens (reviewed in (40)). With the result that deregulated *myc* has been demonstrated to be capable of promoting the development of cancer in each of these tissues. However, due to the time scale and percentage of animals affected it was concluded that deregulated *myc* alone was not sufficient to cause disease (38,39).

The second of these approaches demonstrated the ability of a c-*myc* gene, under the control of the MMTV LTR, to increase the incidence of a variety of tumours (41). Tumour types associated with this multi-tissue overexpression were of ; mammary, testicular, mast cell, pre-B cell, B cell, and T cell origin (41). However, although various cell types developed tumours, these affected only 40% of animals at a mean age of 14 months (41), reinforcing the need for other genetic lesions which enable tumours to develop. Just as intriguing as the role played by *myc* in the various neoplasias, was the finding that the transgenic mice developed normally (41). Hence overexpression does not prevent normal development.

The realisation that deregulated *myc* is insufficient to cause cancers by itself led to other approaches, which utilised either double transgenic technology or retroviral infection to

search for cooperating genetic events in the genesis of tumours. Even mice transgenic for both v-Ha-*ras* and c-*myc* develop clonal tumours, providing evidence that still more events are required to complete tumour development (42). However, this experiment demonstrated that these two oncogenes were synergistic rather than additive in their effects (42).

Although *ras/myc* mice displayed synergy in the genesis of tumours, the most dramatic example to date of a gene which can synergise with *myc* to promote oncogenesis, is *pim*-1. Mice carrying both transgenes succumbed to lymphoblastic leukaemia *in utero* (43). While bitransgenic mice carrying *pim*-1 and either N-, or L-*myc* also succumbed to lymphoid malignancies, these animals survived to a mean age of 36 and 94 days respectively, post parturition (44). Crossing of transgenic mouse strains is now used almost routinely, e.g. recent experiments showed that cyclin D1 (*bcl*-1) which is overexpressed in a variety of human tumours, cooperates with *myc* family members in lymphomagenesis (45,46). This finding, like much associated with *myc*, seems to contradict other work which suggested that highly expressed Myc is able to down-regulate cyclin D1 expression (47,48). However, such a control mechanism might have been lost as the cyclin D1 transgene is under the control of the immunoglobulin enhancer.

Infection with weakly oncogenic retroviruses can promote cancer by insertional mutagenesis, causing inappropriate activation of genes. Using mice transgenic for *myc* and infecting with a retrovirus, cooperating genes may then be cloned and characterised, and this process is termed proviral tagging (reviewed in (49)). This approach has proved to be very effective, leading to the discovery of several other genes involved in carcinogenesis. An early example was *pim*-1 (50), which codes for two primary translation products giving proteins with serine/threonine kinase activity (51). Other cooperating genes include *bmi*-1, a zinc finger protein (52,53), which has been shown

to collaborate with *myc* when transgenic *bmi*-1 mice are mated with transgenic *myc* mice (54).

A technique related to transgenesis is the use of gene knock out, where the gene of interest is deleted in embryonic stem cells by homologous recombination, so affecting the germ line after reconstitution of embryos. By this means heterozygous and homozygous animals deleted for the gene of interest can be generated. This method has been used to generate mice which are null for c-*myc* (55). The resulting homozygous null progeny were found to begin development, but the mutation was lethal by 10.5 days of gestation, while heterozygous females showed reduced fertility with a high rate of embryonic resorbtion (55). The study also demonstrated that c-*myc* was dispensable for early - i.e. before 10.5 days gestation- cellular proliferation and differentiation, although necessary for normal embryonic development.

One further approach has been developed which combines all the previous techniques to search for *myc* cooperating genes, this utilises transgenics, gene knock out and proviral tagging. By crossing Eμ-*myc* mice with mice which are homozygous null for the *pim*-1 gene then infecting with a retrovirus, it was reasoned that insertional mutagenesis would occur at genes which act downstream or in parallel to *pim*-1 (56). This approach, allied to a PCR technique, identified *pim*-2, and has been proposed as a method whereby genes acting in a narrowly defined pathway can be identified by a method termed 'complementation tagging' (56).

1.1.2 Cell cycle progression

When fibroblasts are exposed to growth factors it has been estimated that as many as 100 genes are rapidly induced in the initial response (57). These rapidly expressed genes are thought to be important mediators of mitogenesis. As c-*myc* is one of these immediate early genes, it is supposed that it has an important function in mediating the mitogenic response.

This rapid response in c-*myc* induction has been demonstrated in B cells, T cells, and fibroblasts (58). By using lipopolysaccharide (LPS), concanavalin A (con A), and platelet derived growth factor (PDGF) on B, T, and fibroblast cells respectively, to induce mitosis, it was found that c-*myc* was rapidly transcribed with maximal levels of transcription at approximately 2 hours for lymphocytes and 3 hours for fibroblasts (58). Addition of cyclohexamide did not prevent c-*myc* RNA induction, hence transcription is not secondary to growth and must be mediated by preexisting factors. However, although c-*myc* RNA is rapidly induced when growth arrested cells are stimulated to divide, synthesis of both RNA (59) and protein remain constant throughout the cell cycle (60,61).

Further evidence for the importance of c-*myc* in the mitogenic response came from the use of an exogenous steroid responsive c-*myc* in 3T3 fibroblasts. When c-*myc* was induced by hydrocortisone in density arrested cells, in the presence of epidermal growth factor and absence of PDGF, cells entered the cell cycle as measured by ^{3}H-thymidine uptake (62). Although growth was stimulated along with increased c-*myc* mRNA, it was found that growth was better when PDGF was used to stimulate c-*myc* expression, despite levels of mRNA being similar (62). This was reasoned to be due to factors other than c-*myc* which were not expressed in the absence of PDGF, however another explanation which fits with more recent observations on c-*myc* function, is that the cells entered apoptosis in the poor growth medium (63) (see section 1.1.4).

Rapid downregulation of c-*myc* accompanies removal of mitogenic stimulus or growth inhibition due to cell-cell contact, which has also been demonstrated to suppress the transformed phenotype of v-*myc* transformed quail muscle cells (64). This fits with the notion that abnormal expression of *myc* prevents the cell from leaving the cell cycling compartment, leading to aberrant growth of cells. And c-Myc has been demonstrated to have a direct role in proliferation by microinjection of c-Myc protein into nuclei, which stimulated DNA synthesis when platelet-poor plasma was present (65). Also antisense

nucleotides targeted at c-*myc* were able to inhibit proliferation of human promyelocytic leukaemia HL-60 cells, in a dose dependent and sequence specific manner (66).

Recently a link has been shown to exist which helps to couple the PDGF stimulus to expression of c-*myc*. The non-receptor tyrosine kinase Src binds to the PDGF receptor through the Src-homology 2 domain (SH2). Once bound, Src is activated and can phosphorylate cellular proteins not directly phosphorylated by the PDGF receptor (67). Using a dominant negative Src, lacking kinase activity, it was shown that the PDGF signalling block, induced by the mutant Src, could be rescued by c-Myc, although Myc was unable to rescue a dominant negative Ras (68). Moreover, by using an interfering form of c-Myc it could be demonstrated that prevention of c-Myc function can inhibit DNA synthesis. Further evidence of the importance of the Src pathway was provided by microinjecting antibody which inhibits Src (also Fyn and Yes) and trying to detect c-*myc* mRNA by reverse transcription PCR. This experiment confirmed that SH2-containing kinases directly stimulate c-*myc* transcription as the cells microinjected with the anti-Src antibody had a much reduced level of transcription compared to cells injected with a control antibody (69).

1.1.3 Differentiation block

The ability of c-*myc* to act as a block to differentiation has been demonstrated in a variety of cell types, and a negative correlation exists between c-*myc* transcription and cellular differentiation (70). By using teratocarcinoma stem cells, which differentiate into nonproliferating endoderm by addition of retinoic acid and cyclic AMP (RA+cAMP), c-*myc* mRNA levels were 15 to 20 fold lower three days after addition of RA+cAMP (71). This striking reduction in c-*myc* mRNA correlated with terminal differentiation before proliferation had ceased (71).

Mouse erythroleukaemia (MEL) cells can be induced to differentiate by using DMSO, lowering c-*myc* mRNA levels within two hours, with recovery to normal levels after 24 hours (72). Constitutive expression of c-*myc* by using vectors expressing the gene

under the control of the SV40 early promoter, prevented MEL cells from differentiating when treated with DMSO (73). Using a similar SV40 driven construct, but with c-*myc* in the antisense orientation, differentiation of MEL cells was accelerated while G_1 progression was inhibited (74).

It appears that the c-*myc* induced block to differentiation may be caused by preventing cells from entering a predifferentiation compartment in G_0/G_1. A preadipocyte cell line (3T3-L1) expressing c-*myc* under the control of a Rous sarcoma virus promoter, was able to proceed through the cell cycle and growth arrested in G_0/G_1, as did non c-*myc* overexpressing 3T3-L1 cells. However, unlike normal cells, *myc* expressing cells could re-enter the cell cycle, and failed to terminally differentiate on addition of high concentrations of serum (75). The differentiation block was reversible when the same cells were induced to express c-*myc* antisense RNA from a methotrexate inducible gene. It is proposed that c-*myc* acts as a molecular switch directing cells either to terminal differentiation or continued proliferation (75).

However, as with other aspects of c-Myc function, any ability to block differentiation may be specific to a particular experimental design. Evidence comes from transgenic mice carrying either c-*myc* or L-*myc* under the control of the αA-crystallin promoter. In this system expression is targeted to the lens fibre compartment, and was used to show that continued expression of c-*myc* does not directly prevent differentiation of this cell type, although there was inappropriate cell cycle progression (76). In contrast, continuous expression of L-*myc* had a pronounced effect in preventing the expression of late-stage differentiation markers, however these cells could withdraw from the cell cycle (76).

1.1.4 Apoptosis

As recently as 1990 major cancer meetings viewed oncogenesis as a failure to prevent cellular proliferation (77), with the focus of research on oncoproteins directed at two principal modes of action, (i) regulation of gene expression, and (ii) the control of

cellular proliferation (78). The review of the 1990 Origins of Human Cancer meeting held at Cold Spring Harbor, is also a good example of changing fashions in science:

"If this meeting had been held three years ago, it safe to predict that attention would have been overwhelmingly focused on oncogenes: mutant genes that promote abnormal cellular proliferation. However, it has become increasingly clear that other genetic elements, namely tumor (sic) suppresser genes, play a critical role in negatively regulating cellular proliferation" (77).

Although both tumour suppresser genes and oncogenes are studied in even more depth today, the focus of oncogenesis has shifted with a current upsurge of interest in programmed cell death, or apoptosis (for reviews see (79-83)).

Apoptosis is a well recognised phenomenon which occurs during embryogenesis as part of the modelling of tissues, as well as being required for tissue homeostasis, characterised by cytoplasmic blebbing, vesicularization, nuclear condensation, and DNA fragmentation (84). The concept of natural cell death as a means of tissue modelling was first developed in the middle of the nineteenth century, with the first clear morphological description in 1885 given the name 'chromatolysis', while this type of cell death was reported in breast cancer in 1892 (85). So although the term apoptosis may be a relatively recent epithet, the process was observed over 100 years ago.

Present day thinking on the role of oncogenes and tumour suppresser genes tends more and more to look at how a particular gene product can either promote or prevent apoptosis. Now cooperation between oncogenes is no longer viewed as promoting mitogenesis, rather genetic mechanisms which can inhibit apoptotic pathways are deemed to be at least as important (86,87). Much of this refocusing of cancer research has been stimulated by the finding that constitutive overexpression of *myc* can lead to apoptosis in certain circumstances, such as serum deprivation in fibroblasts, which would undergo growth arrest if excess *myc* was not available (63). In haematopoetic cells loss of IL-3 in myeloid cells leads to apoptosis when *myc* is ectopically expressed (88), and T-cells fail to undergo activation induced apoptosis when antisense *myc*

oligonucleotides are present, the latter finding suggesting a role for *myc* in the negative selection of T-cells (89).

Further work has looked at cooperation between *myc* and other oncogenes and tumour suppresser genes. Foremost amongst these are *bcl*-2, which can prevent cell death and is localised on the inner mitochondrial membrane (90-92), and p53, a gene thought to be the most commonly mutated in human cancers (93). Initial work focused on cooperation between *myc* and *bcl*-2 in tumour induction (90,94), however the mode of this cooperation was soon under investigation.

Using a heat shock inducible *myc* in Chinese hamster ovary cells, it was demonstrated that a constitutively expressed *bcl*-2 increased cell viability compared to parental cell lines which underwent apoptosis (95). Also, a c-Myc-oestrogen receptor construct which is able to induce apoptosis in fibroblasts when β-oestradiol is added to serum deprived fibroblasts, lacks this effect when *bcl*-2 is constitutively expressed (96). Further, it was demonstrated that *bcl*-2 expression did not inhibit cellular proliferation, and therefore *bcl*-2 is a specific inhibitor of the *myc* induced apoptosis function (96). This work helped to formulate the 'two signal' model in which c-Myc can provide the first signal leading either to cell cycle progression or apoptosis, while the second signal could be provided by, e.g. growth factors which inhibit apoptosis, allowing c-Myc to drive the cells into cell cycle (63,95).

Confirmation of the role played by c-Myc in the induction of apoptosis was achieved by the demonstration that heterodimerization was required. This was accomplished by the creation of leucine zipper mutants of Myc and Max which could dimerize efficiently with each other, but not with their wild type partners (97). Only coexpression of both Myc and Max mutants in the same cells caused apoptosis to occur on withdrawal of serum (97). The same approach demonstrated that the activation induced apoptosis of T-cell hybridomas was dependent on functional Myc heterodimers (98).

With the finding that the tumour suppresser p53, in its wild type form, is required for c-Myc mediated apoptosis, a link between the transforming capacities of these two genes was established (99). Using a β-oestradiol-inducible c-Myc, it was found that wild type p53 was stabilised after addition of β-oestradiol, cells then underwent apoptosis when deprived of serum and β-oestradiol was present. However, apoptosis did not occur in cells lacking p53 (99). Further evidence for the role of p53 in apoptosis, comes from Burkitt's lymphoma cells which express mutant p53, and grow rapidly in culture. However, when the same cells expressed a temperature sensitive wild type p53 they showed a diminished growth potential at a temperature which was permissive for the exogenous p53 (100). This loss of growth potential was shown to be due to the induction of apoptosis in cells expressing p53 in its wild type conformation (100).

In contrast, although p53 seems to be required for *in vitro* Myc-induced apoptosis, it appears to be dispensable for apoptosis *in vivo*. Crossing of Eμ-*myc* mice with p53 heterozygotes led to a synergistic shortening of tumour latency, with most tumours losing their wild type p53 (101). But it was found that tissue sections of tumours from Eμ-*myc* mice and Eμ-*myc* mice lacking a wild type p53, displayed equivalent levels of apoptosis, suggesting that wild type p53 is not required for apoptosis in this system. One problem with this study is that the apoptosis displayed within the tumour tissue may be regulated by factors other than Myc and p53, as p53 independent pathways exist (83).

1.2 The c-*myc* Gene in Feline T-cell Lymphomas

Viruses were implicated in the genesis of cancer long before the structure and life cycle of the transforming retroviruses was discovered. Ellerman and Bang in 1908 transmitted erythroleukaemia and myelogenous leukaemia by inoculating chickens with cell-free filtrates (12). And Rous in 1911, who gave his name to the Rous sarcoma virus (RSV),

used cell-free extracts from chicken sarcomas to transmit the same disease to a normal animal (12). The discovery of feline leukaemia virus owed much to the same methodology. A virus was isolated from a cat with lymphosarcoma in a multi-cat household, where other animals had developed the disease, and demonstrated to be an exogenous retrovirus transmitted contagiously among cats (91,102).

The majority of spontaneous lymphoid tumours which arise in the domestic cat are associated with feline leukaemia virus (FeLV) infection, with tumours falling into two major groups: mainly virus-positive T-cell lymphosarcomas, and alimentary B-cell lymphosarcomas, which are mainly virus-negative (13).

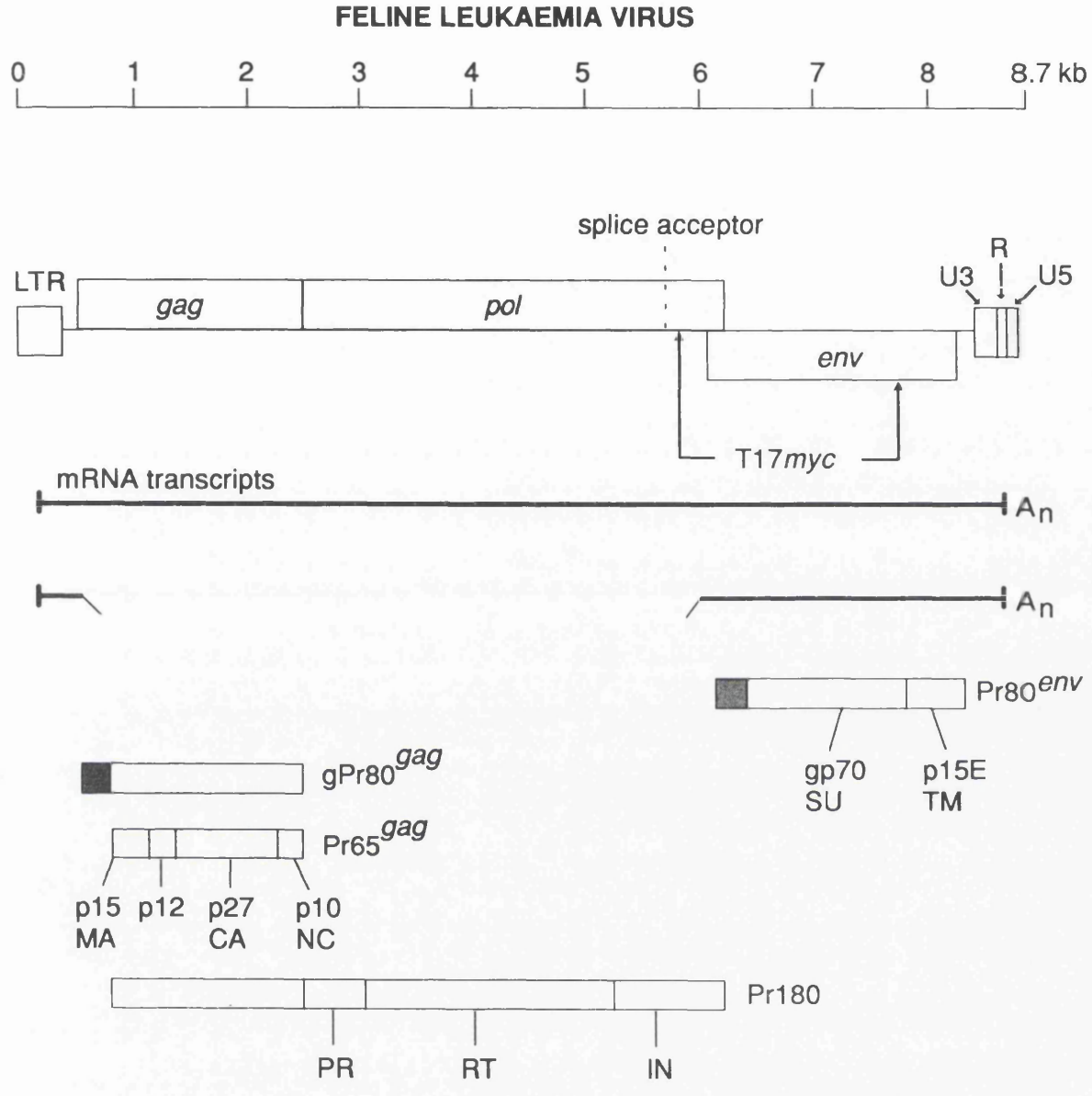
FeLV is an archetypal 'simple' retrovirus with three main open reading frames encoding *gag*, *pol* and *env* (103). The *gag* gene codes for internal antigens of the virus, *pol* codes for the reverse transcriptase, and *env* codes for the glycoproteins which are present in the envelope (Figure 1.1).

Although most of the work carried out on FeLV has concerned the development of neoplasias, the majority of cats actually die of other non-neoplastic degenerative disorders (Table 1.1, from (104)).

Table 1.2 Diseases induced by FeLV

Neoplastic	**Degenerative**
T cell lymphosarcoma	Immunosuppression
B cell lymphosarcoma	Pure red cell aplasia
Acute lymphoblastic leukaemia	Aplastic anaemia
Myeloid leukaemia	Osteosclerosis
Multicentric fibrosarcomas	Abortion and foetal resorbtion

FeLV aetiology of lymphoid tumours is associated with *myc* in approximately 1/3 of field cases (13), where the virus affects *myc* in one of two ways. Either the virus can integrate near to cellular *myc*, so activating the gene via the viral LTRs (13), which appears to upregulate the gene only marginally, leading to the suggestion that upstream sequences regulating the normal feedback control of *myc* expression may be disrupted. Or, more commonly, the virus transduces the protein coding second and third exons of c-*myc*. This latter mechanism results in deletion of part of the viral genome (13,105,106), rendering the viruses replication incompetent.



Legend for Protein Nomenclature-

SU = surface; TM = transmembrane
MA = matrix; CA = capsid; NC = nucleocapsid
PR = protease; RT = reverse transcriptase; IN = integrase

Figure 1.1

Figure 1.1 Structure of FeLV. Protein nomenclature after Leis *et al* (107).

However, although it is c-*myc* which is transduced by FeLV in lymphoid tumours, there is a different picture in fibrosarcomas. Listed in table 2 are a number of other genes transduced by FeLV, but these genes were found in fibrosarcomas, transduction of a cellular proto-oncogene by FeLV forms a feline sarcoma virus (FeSV) *de novo* (103). It seems that FeLV transduction of cellular genes is actually a very rare event as all the transduced genes listed have been found in field cases, and no *in vitro* nor *in vivo* experiment has been able to reproduce transduction any of these genes (108).

Table 1.3 Fibrosarcoma derived FeLV transduced host genes (adapted from (108)).

Isolate	Host gene	Gene Function
FeSV-HZ2	*abl*	Non-receptor tyrosine kinase
FeSV-GA	*fes*	
FeSV-ST	*fes*	Non-receptor tyrosine kinase
FeSV-HZ1	*fes*	
FeSV-GR	*fgr*, actin	Non-receptor tyrosine kinase
FeSV-TP1	*fgr*	
FeSV-SM	*fms*	Tyrosine kinase derived from
FeSV-HZ5	*fms*	CSF-1 receptor
FeSV-HZ4	*kit*	SCF receptor tyrosine kinase

Gene functions taken from (109).

CSF, colony stimulating factor; SCF, stem cell factor.

Intriguingly the T-cell tumours which arise through FeLV-*myc* viruses can usually be established *in vitro* without the requirement for exogenous interleukin-2. Allied to this is the late developmental stage of the tumours, which express both the α-, and the β-chains of the T-cell antigen receptor (110). However, it is not clear if tumours actually arise late in ontogeny, or whether the transformed phenotype occurs earlier in development and the cells continue to differentiate.

It appears that retroviruses capture the cellular proto oncogene by recombining with cellular DNA. The original proposal was that the retrovirus integrates 5' to the cellular gene which gets transduced. Subsequently a deletion gives a viral-cellular gene fusion transcript, which can be spliced and packaged into a virion. A further recombination event between the chimaeric RNA and a full length viral RNA supplies the 3' end of the recombinant viral genome (111). Experimental evidence used defective proviruses which carried a transduced *ras* and only one LTR (intact), or a U3 deleted LTR. The intact 3' LTR is necessary for replication but not transcription (112), and so proviruses with the U3 deletion would only be able to recombine at the DNA level, rather than by RNA template switching. Therefore the finding that all the constructs transformed cells and produced identical RNAs, but only the replication competent single LTR produced infectious virus, when helper virus was available, points to recombination at the DNA level (113).

1.3 Structure and Deduced Function of c-Myc

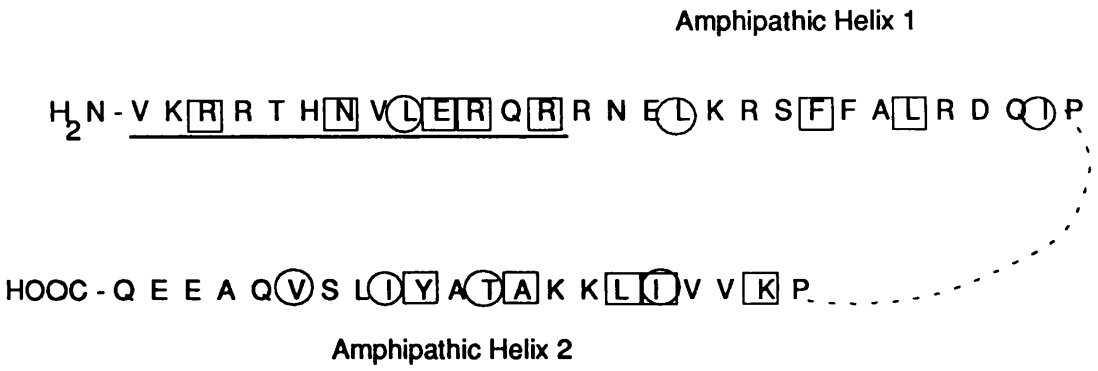
The Myc protein is thought to be a transcription factor from the structural motifs which it shares with established transcription factors. These motifs are; a basic region of around 30 amino acids, followed by a helix loop helix and a leucine zipper domain, plus a transactivation domain at the N-terminus (7). The basic region confers sequence specific DNA binding to a 5'-CANNTG-3' "E box" motif, in the case of Myc the optimal sequence is 5'-CACGTG-3' (114). Helix loop helix and leucine zipper domains allow protein dimerization, this can be as homodimers or heterodimers, though Myc does not form homodimers under physiological conditions, but it does form a heterodimeric complex with another bHLH-Zip protein known as Max (115).

1.3.1 Helix-Loop-Helix

The Helix-Loop-Helix motif consists of a ~15 amino acid sequence giving an amphipathic alpha-helix, followed by a region of varied length, e.g. 9 amino acids in c-Myc and up to 28 amino acids in related *Drosophila* proteins (the "loop"), before another ~15 residue helix (116,117). The HLH domain allows dimerization, and increases the affinity of DNA binding. However, binding to DNA is the main function of a highly basic region (BR) of up to 30 amino acids, which is rich in arginine and lysine (118). The basic region lies immediately N-terminal to the HLH and confers sequence specific DNA binding (7). With a recognition sequence which follows the pattern 5'-CANNTG-3', where N is any base, these bases are found in the target sequences of many other BR-HLH transcription factors that bind to the E box motif of transcriptional enhancers (7).

The BR-HLH proteins have various roles in development and differentiation, with controls exerted by heterodimer formation. e.g. The MyoD protein, which appears to be the primary signal for the differentiation of muscle cells, binds DNA greater than ten times more tightly when forming a heterodimer with a ubiquitously expressed E2A protein splice variant (119).

Amino acid sequence alignment of transcription factors shows that there are high degrees of homology (Fig.1.2). Experiments have been conducted to ascertain the functional significance of these similarities, such as Fisher and Goding's work (120), where the basic region of c-Myc was substituted for the basic region of the yeast transactivator PHO 4, which recognises the same consensus motif as Myc, leading to transactivation of PHO 5. This provided proof that the primary amino acid sequence can be a good predictor of function.



Boxes = Amino acids conserved between members of the family.
Circles = Always hydrophobic, allowing formation of two alpha helices.
Dotted line = the variable length loop
Underlined = Basic Region

Fig. 1.2 bH-L-H domain of c-Myc

Although figure 1.2 shows residues proposed to make up the c-Myc BR-HLH domains, it should be noted that these domains do not have well defined boundaries. Therefore I have used several sources to construct this figure (116,121-123).

1.3.2 Leucine Zipper

Leucine zippers (LZ) were first identified by homology studies with a wide range of factors, including yeast GCN 4 and mammalian CAAT/enhancer binding protein (C/EBP), as well as the Fos and Jun proto oncogene products (124). Sequence homology showed there was, again, a stretch of approximately 30 amino acids, with a substantial net basic charge, immediately followed by a region containing, usually, four leucine residues positioned at intervals of seven amino acids. It was this latter region that has been termed the leucine zipper (124), and is proposed to be a coiled coil with two parallel α-helices (125). A coiled coil has a periodicity of 3.5 residues per helical

turn, placing the heptad repeat of leucines on the same side of the molecule (118). High aqueous solubility of the GCN4 zipper region suggested that the hydrophobic side chains are shielded from solvent in a closely packed interface between the two helices (125). A leucine zipper protein can dimerize with other proteins containing the requisite leucine zipper motif. In fact to act as transcription factors these proteins must dimerize, either as parallel homodimers (e.g. JUN : JUN), or as heterodimers (e.g. FOS : JUN), via the leucine zipper (126).

The FOS and JUN proteins also illustrate the cooperative nature of dimerization. Although JUN : JUN homodimers can bind the AP-1 binding site (FOS does not appear to form stable homodimers) the affinity is relatively low. However, it can be shown, using transcriptional transactivation assays, with reporter genes fused to AP-1 binding sites that FOS and JUN act synergistically (126).

The suggested model of the zipper structure is again an amphipathic alpha-helix. Cytosine oxidation studies, nuclear magnetic resonance, and X-ray crystallography, have all provided evidence that the GCN 4 dimerization region can form a parallel coiled-coil structure (125,127). These studies led to the proposal that BR-LZ dimers are Y shaped complexes, with the leg of the Y denoting the LZ dimerization domain (Fig.1.3). The arms of the Y represent the amino terminal basic regions, able to contact the specific recognition sites in the major groove of the DNA.

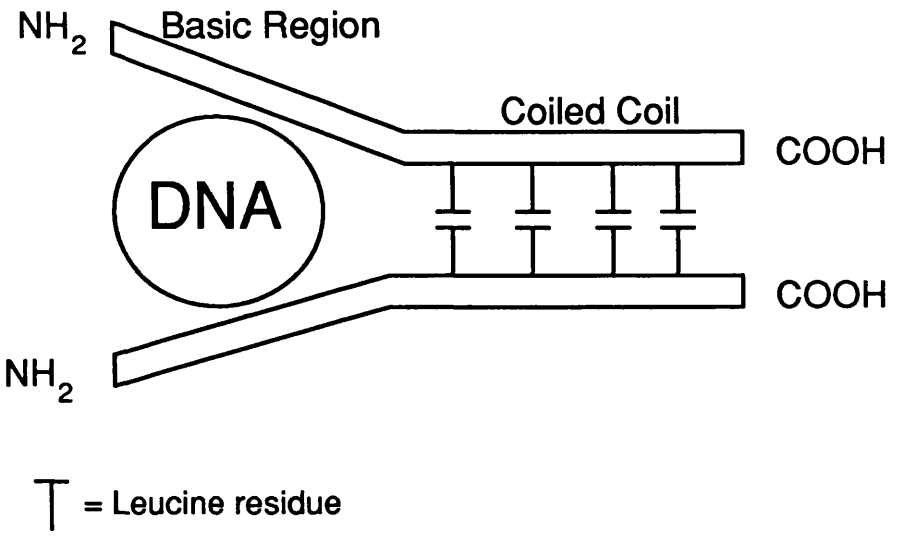


Figure 1.3. Hypothetical BR-LZ dimer structure (127). This one dimensional representation cannot display the left-handed coiled-coil nature of two right-handed α-helices, which make up the zipper domain of each monomer (128).

Due to the conserved motifs present in c-Myc and its apparent inability to form homodimers (129), a search was made to find a heterodimeric partner of c-Myc. Using ^{125}I-labelled BR-HLH-LZ c-Myc fusion proteins purified from bacteria to screen a complimentary DNA expression library, colonies were identified which encoded a small novel protein which was named Max (115). Both c-Myc and its heterodimeric partner Max contain a basic region followed by both an HLH and a LZ (115), although the LZ is not preceded by a basic region (figure 1.4). It has been shown that Max is an obligate partner enabling Myc to bind to DNA (130). Myc and Max also demonstrate a further level of transcriptional control, since the binding of Max to Myc, which is required for Myc binding to DNA, can be prevented by other factors, such as Mxi 1 and Mad family proteins (122,123,131). Mxi 1 and Mad,1,2,3 and 4 are also bHLH LZ proteins which form DNA sequence-specific binding complexes, in association with Max. Transactivation assays demonstrate that Mad and Mxi 1 can prevent Myc/Max mediated transcription, by sequestering Max, thereby preventing formation of Myc/Max heterodimers, and/or binding to Myc/Max target sites (122,123). However, this simple

model of Mxi1 and Mad repression of Myc mediated transcription has recently been called into question, with the discovery that isoforms of Mxi1 and Mad can interact with a mammalian homologue of the yeast transcriptional repressor SIN3 (132,133).

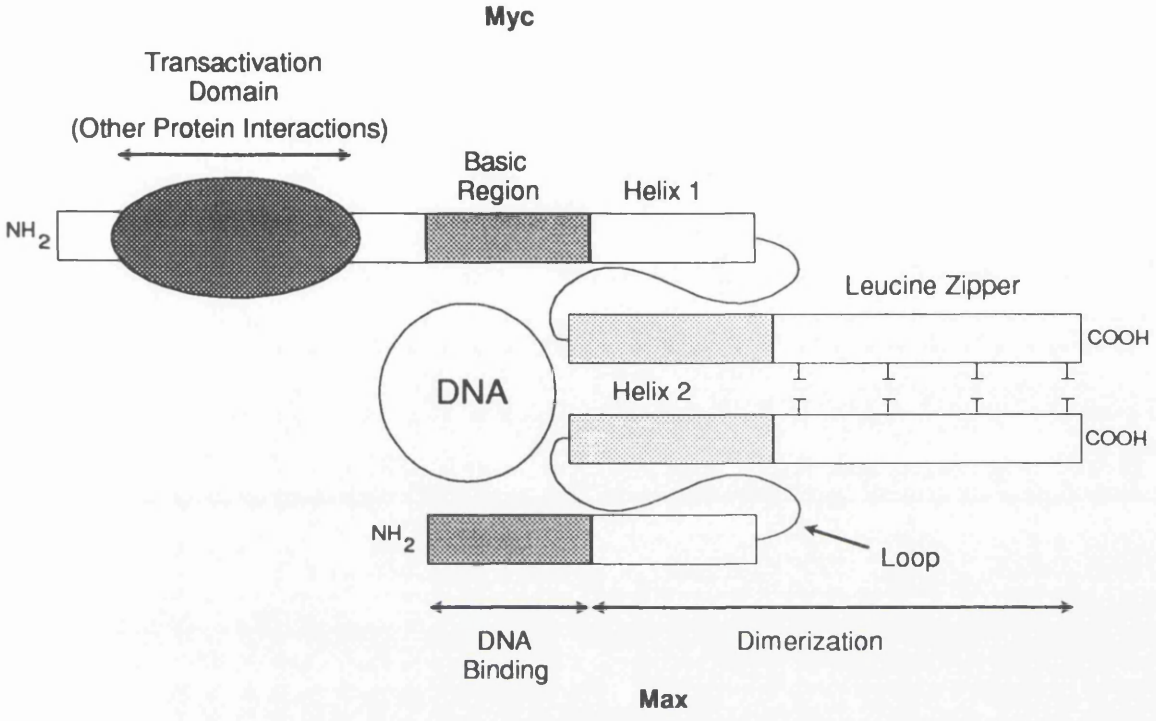


Figure 1.4 Diagram depicting the heterodimeric Myc/Max pair. Adapted from (127)

1.3.3 Transactivation Domain

Protein activation domains are separable from their associated DNA-binding activities, and a single protein can have more than one activation domain, with any single domain spanning from 30 to 100 amino acids (134). The first defined activation domain of a eukaryotic transcription factor, was from the yeast factors GAL 4 and GCN 4. Common features were the ability to form amphipathic alpha helices and a significant negative charge. GAL 4 has two separate acidic activation domains, which can activate transcription from reporter genes when linked to a heterologous DNA-binding domain (135). A correlation also exists between activation and negatively charged alpha-helical regions in the JUN transcription factor (136).

A second type of activation domain is that found in the Sp1 Zinc-fingered protein. Deletion analysis of Sp1 showed four separate regions involved in activation. The two most potent contain about 25% glutamine and few charged residues. Comparison with other transcription factors showed glutamine rich regions in, amongst others, *Drosophila* Zeste and Ultrabithorax; yeast Gal 11 and Hap 1; and in mammalian Oct-1, Jun, and AP-2 (134). Again activation domains appear to be somewhat interchangeable, since a glutamine-rich stretch of 145 amino acids from *Drosophila* antennapedia was able to partly substitute for the activation domain when linked to Sp1 Zinc fingers (137). Both glutamine rich and acidic domains are only similar to other like-proteins, by virtue of their glutamine content and their acidity, respectively. i.e. there is no obvious primary sequence homology (134).

A third kind of activation domain is proline-rich, e.g. in CTF/NF1, a domain containing up to 30% proline residues in the C-terminus. This domain activates transcription when linked to various DNA binding domains, including the Zinc fingers of Sp1. Regions rich in proline have been recorded in other mammalian transcription factors, including AP-2 and OCT-2 (134).

The N-terminal domain of c-Myc has been classed as a transactivation domain by analogy with homologous regions of established transcription factors. Approximately 15% of the N-terminal 143 amino acids of Myc are acidic, while another 15% are made up of proline and glutamine. Moreover, this region of the N-terminus has been shown to be capable of activating transcription when linked to a heterologous promoter (138).

However, unlike homology between HLH and LZ domains, where there is a definite structure conferred by the primary amino acid sequence, transactivation domains appear less well defined (118,139). The common features include a net negative charge and possibly the ability to form a helical structure. Also pertinent is the observation that incorporation of proline, glycine, or serine can disrupt secondary structure, and these

amino acids are highly represented in c-Myc, leading to the conclusion that the tertiary structure of c-Myc could contain several solvent accessible loops (140). These loops in the N-terminus of c-Myc would then be available for interaction with other proteins.

More evidence that Myc is a transcription factor comes from assays in yeast cells. These are used as an *in vivo* system in which transactivation can be measured without the complication of endogenous Myc or Max interfering with the result. This method was used to demonstrate that Myc and Max were required to activate transcription from a reporter plasmid containing LacZ (141). The same study showed that Max has a higher affinity for Myc than for itself.

1.4 Other Evidence That c-Myc is a Transcription Factor

The minor p67 form of cMyc is often absent from tumours due to loss of exon 1 sequences, but is found to reach levels just as high as the p64 form when tissue culture cells reach high density (142). This phenomenon could be replicated by treatment of cells at low density with conditioned media from cells at high density. Amino acid deprivation, specifically methionine deprivation, was shown to be responsible for the control mechanism (142). It has been postulated that the two proteins possess distinct transcriptional activities, due to different abilities to activate transcription from a Rous sarcoma virus long terminal repeat (LTR) EFII enhancer element in COS cells (143). Whereas p67 Myc could initiate transcription from the EFII enhancer, the major p64 form was unable to do so, although both forms were able to transactivate a canonical Myc/Max binding site (143). Evidence for functional similarity came from experiments where both p64 and p67 Myc could transform Rat-1 fibroblasts in conjunction with *bcr-abl*, and were able to activate transcription of a Myc/Max reporter construct to similar levels (144). The latter set of experiments gave rise to the hypothesis that the CUG

translational initiation of *myc* is a mechanism whereby Myc is synthesised when AUG initiation is inhibited (144).

Recently there have been several reports of c-Myc interacting directly with elements of the transcriptional machinery, including the TATA binding protein (TBP) (145,146), a zinc finger protein called Yin-Yang-1 (YY1)(147), transcription factor II-I (TFII-I) (148), and the retinoblastoma gene product related gene p107 (149).

1.4.1 TBP

The first of these proteins, TATA binding protein, is part of the general transcription machinery (transcription factor II D, TFII D) which binds to the sequence TATAAA, at a position -25 to -30 relative to the RNA transcription start site, and may be required for all RNA PolII transcribed genes (150,151). Use of *in vitro* binding assays demonstrated that the N-terminal 204 amino acids of c-Myc could interact with TBP (146)), while immunoprecipitation was used to demonstrate that c-Myc was complexed with TBP in cell lysates (145,146).

1.4.2 YY1

Yin-Yang-1 (YY1) is a zinc finger protein (152) which has been reported both to repress and activate transcription (152-154). In the yeast two-hybrid system c-Myc could interact directly with YY1, and *in vitro* binding assays demonstrated that amino acids 250-439 of c-Myc mediated this binding most strongly (147). The same work demonstrated that c-Myc could both prevent the repression and activation of reporter constructs designed to test these YY1 functions (147).

Intriguingly YY1 has been reported to mediate transcription of c-*myc* itself. When YY1 was expressed under the control of cytomegalovirus promoter and enhancer, a reporter

plasmid containing the c-*myc* promoter linked to the luciferase gene was activated, but mutation of the YY1 zinc finger domain negated the activation (155). When the same YY1 expression plasmid was transfected into murine erythroleukaemia cells c-*myc* RNA levels were increased (155). These findings suggest a possible regulatory loop, where c-Myc negatively regulates its own transcription via YY1. This hypothesis could account for the observation that c-Myc can negatively autoregulate its own expression in a dose-dependent, and reversible way (156). The autoregulatory loop described did not involve a simple interaction between c-Myc and c-*myc* regulatory sequences (156), and other work has reinforced the idea that autosuppression works by an indirect route (157). These observations would be accounted for if YY1 were part of the loop. However, it is not clear if such a mechanism could account for the apparent silencing of the normal *myc* allele in Burkitt lymphomas and mouse plasmacytomas (23), or whether methylation of the untranslocated allele is the cause (158). Silencing of the normal c-*myc* allele(s) may well be outwith the direct control of c-Myc, as ectopic expression did not suppress endogenous c-*myc* expression of subconfluent cells in culture, although the cellular gene was shut off in tumours caused by these cells when injected into nude mice (37).

1.4.3 TFII-I

Transcription factor II-I is an initiation factor which activates core promoters through a sequence termed the initiator element (Inr) (159). The ability of c-Myc to interact with TFII-I has been demonstrated to inhibit transcription mediated by TFII-I in a dose dependent manner (148). This negative regulation by c-Myc was shown to require the HLH and LZ domains of c-Myc, whereas an extensive N-terminal deletion had no effect, and is mediated by c-Myc preventing TBP-TFII-I promoter complex formation (148). It is postulated that c-Myc inhibition of the Inr is the method by which c-Myc negatively regulates cyclin D1 gene expression (48).

1.4.4 p107

p107 bears homology to the retinoblastoma gene product pRB, especially in the 'pocket region' which is critical for the association of adenovirus E1A, and SV40 large T antigen with both p107 and pRB (160). E1A and large T mediate their effects on p107 and pRB by dissociating complexes formed between the transcription factor E2F and p107 or pRB (161). p107 has been demonstrated to inhibit cellular proliferation, and can repress transcription via interaction with E2F (161). Direct interaction between c-Myc and p107 has also been reported, in this case it is again the N-terminal of c-Myc which has been implicated as the site of interaction, suppressing c-Myc transcription of reporter constructs (149,162). Enforced expression of c-Myc can release cells from growth arrest induced by p107 (162). Perhaps of greater importance, c-Myc mutants derived from Burkitt's lymphoma cells were not suppressed by p107 in transactivation assays, although the Myc/p107 association was still detected by immunoprecipitation (149). Together these data provide another direct link between c-Myc and transcriptional regulation within the nucleus.

1.5 Possible Target Genes Under the Direct Transcriptional Control of c-Myc

Although there is a great deal of evidence that c-Myc is a transcription factor, there is still a paucity of evidence on genes which are transactivated or transrepressed directly by c-Myc. However, there are several candidate genes, including; *cad* (163); ECA39 (164); p53 (165); prothymosin-α (166); ornithine decarboxylase (167-169), and cyclin D1. Both prothymosin-α and ornithine decarboxylase are dealt with as c-Myc target genes in chapter 8.

1.5.1 *cad*

The carbamoyl-phosphate synthase (glutamine-hydrolysing)/aspartate carbamoyltransferase/dihydrooratase (*cad*) gene product is a trifunctional enzyme catalysing the first three steps in the *de novo* synthesis of pyrimidines. The enzymatic activity and mRNA of *cad* correlate with the proliferative state of the cell (163). Nuclear proteins which bound to a 5'-CCACGTGG-3' E-box sequence centred at +65 of the untranslated sequence of the *cad* gene in electrophoretic mobility shift assays (EMSA), were found to be inhibited by anti-Max antibodies (163). Mutation of the E-box motif abolished growth dependent transcription from a luciferase reporter construct, while expression of dominant negative mutants of c-Myc, lacking either a basic region or transactivation domain, inhibited *cad* transcription during G_1/S-phase induction (163)

1.5.2 ECA39

The ECA39 gene was identified as a target for Myc/Max regulation in a differential screen
of normal brain tissue and tissue from a c-*myc* transgenic mouse brain tumour (164). ECA39 is of unknown function and has a Myc/Max binding site 3' to the transcriptional start site, this sequence was found to bind a nuclear protein complex which included c-Myc. Transfection of c-*myc* was shown to block the normal downregulation of ECA39 which occurs as embryonic stem cells undergo differentiation (164).

1.5.3 p53

Initially a BR-HLH 5'-CANNTG-3' consensus motif at position +70 to +75 of the p53 gene, was demonstrated as being responsible for the binding of nuclear proteins, and use of oligonucleotide competitors in an EMSA demonstrated that the actual sequence was 5'-CACGTG-3' (170). Myc/Max translated *in vitro* were shown to bind to oligonucleotides with a sequence derived from the murine p53 gene in an EMSA(165). Cotransfection of c-*myc* and a reporter construct containing the *E. coli* chloramphenicol acetyltransferase gene linked to the p53 promoter region, stimulated transcription from

the reporter plasmid, whereas no stimulation of transcription was detected from cotransfection with wild-type p53, MyoD, or puc 19. Furthermore, cotransfection of modified c-*myc* constructs which could not dimerize with Max or were unable to bind DNA did not stimulate transcription from the reporter plasmid (165).

1.5.4 Cyclin D1

Cyclin D1, like other cyclins, is a regulatory subunit of a cyclin-dependent protein kinase complex, and is normally expressed during the G_1 interval of the cell cycle (171). Cyclins act as positive regulators of cellular proliferation by linking growth factor mediated signals to the decision of a cell to enter the cell cycle (171). Constitutive expression or activation of c-Myc-ER chimaeras was found to repress cyclin D1 expression, although cyclins A and E were increased 8-fold and 3-fold respectively (47). Further work showed that amino acids 92 to 106 were necessary for the Myc-mediated repression, and that using an insertion mutant which fails to heterodimerise with Max *in vitro*, repression was unaffected. Hence heterodimerisation was not required for the repression of cyclin D1 (48). However, the tendency of research on c-Myc to obfuscate was maintained with regulation of cyclin D1, as use of the Myc-ER system has also been demonstrated to rapidly induce cyclin D1 expression within 30 minutes of the addition of oestrogen (172).

1.6 Aims of This Study

This project is centred on a particular v-*myc* gene transduced by feline leukaemia virus found in a field case of lymphosarcoma(173). The transduced v-*myc* was found to carry several novel mutations which suggested that it might provide a tool for dissecting the mechanisms of action of *myc* in the genesis of tumours.

CHAPTER 2

Chapter 2

Materials and Methods

2.1 Materials

Commonly used reagents and equipment are outlined below.

2.1.1 Chemicals and Enzymes

Chemicals were obtained from the Sigma chemical company, BDH chemicals, Boehringer Manheim, Pharmacia or Gibco BRL, except where otherwise stated. All chemicals were of Analar quality. Enzymes unless otherwise stated, were supplied by Gibco BRL along with appropriate buffers.

2.1.2 Radiochemicals

Supplied by Amersham UK Ltd., except (γ-^{32}P) ATP which was supplied by ICN Flow Biomedicals.

2.1.3 Media and Antibiotics

L-broth : 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride in ddH_2O autoclaved and stored at room temperature. Ampicillin was used at a concentration of 50µg/ml as required.

L-agar : as for L-broth, but also containing 1.5% (w/v) agar, again ampicillin was added to a concentration of 50µg/ml as required.

2.1.4 Cloning Vectors

pCR II: *lacZ*$^+$, *amp*R$^+$, *kan*R$^+$, plasmid designed for the specific cloning of PCR products, by making use of the non-template dependent addition of a single deoxyadenosine to the 3'-ends of duplex DNA molecules catalysed by *Taq* polymerase (Invitrogen).

pSFCV-LE sa$^+$: *amp*R$^+$ containing the LTR's and *gag* gene from the avian erythroblastosis virus (*pol* and *env* are deleted) and the *neo* resistance gene, used to transfect primary chick cells (174).

pGEX: *amp*R$^+$ pBr322-derived plasmid which carries part of the *Schistosoma mansoni* Glutathione S-transferase (GST) gene (175).

pMA132 P4: *amp*R$^+$ yeast vector containing the sequence for the transactivation domain of PHO4 controlled by the PGK promoter (176).

pRS314: *amp*R$^+$ yeast vector allows transcription directed by a GAL promoter (176).

pV44.Lex.Bgl II: *amp*R$^+$ GAL inducible yeast vector with sequence for the LexA DNA binding domain downstream of a CYC promoter (177).

pKV701: *amp*$^+$R yeast vector giving induction from a *GAL10* promoter (120).

2.1.5 Bacterial Strains

E. coli DH5α: F$^-$, ϕ80d*lac*ZΔM15, *end*A1, *rec*A1, *hsd*R17 (r_K^-, m_K^+), *sup*E44, *thi*-1, *deo*R, *gyr*A96, *rel*A1, (Δ*lac*ZYA-*arg*F), U169, λ$^-$. (Supplied as competent cells by Life Technologies Inc., GIBCO BRL.)

E. coli INVαF: *end*A1, *rec*A1, *hsd*R17(r^k, m^{+k}), *sup*E44, λ$^-$, *thi*-1, *gyr*A, *rel*A1, ϕ80*lac*ZΔM15Δ(*lac*ZYA-*arg*F), *deo*R+, F' (Supplied as competent cells by Invitrogen, with the TA cloning kit.)

2.1.6 Eukaryotic Cells

Chick Embryo Fibroblasts: these cells were derived from 10 day old chick embryos, and were the kind gift of Dr. D.H. Crouch and Mr. Billy Clark, of the Beatson Institute for Cancer Research.

3201: suspension cells derived from a feline lymphosarcoma. These cells are FeLV negative but have a germ line rearrangement of c-*myc* (178)

F422: feline T-cell line which contains an FeLV transduced full length c-*myc*, established from a lymphosarcoma of a kitten inoculated with the second passage of the Rickard strain of FeLV (179).

T17: cell line derived from the original tumour, containing both transduced c-*myc* and transduced T-cell receptor β-chain, as well as helper virus (173).

AH927: a feline fibroblast cell line, derived from a feline embryo culture which underwent spontaneous transformation (180).

2.1.7 Yeast Strains (*Saccharomyces cerevisiae*)

Y704: α, *ade2*-1, *trp1*-1, *can1*-100, *leu2*-3, *leu2*-112, *his3*-11,15, *ura3*, *pho4:HIS3* (120).

2.1.8 Stock Solutions

Ammonium persulphate: 10% W:V stock solution, freshly made as required.

Ampicillin (500X): 25mg/ml in ddH_2O. Filtered through 0.22μm filter, aliquoted and stored at -20°C.

Bradford's Reagent (1X): 0.1mg/ml Coomassie Blue G, 1:20 V:V 95% EtOH, 1:10 V:V Orthophosphoric acid in ddH_2O. Stored in dark glass at room temperature.

Denaturation Buffer: 1.5M NaCl, 0.5M NaOH in ddH_2O. Stored at room temperature.

Denhardt's Solution (50X): 1% Bovine Serum Albumin (BSA), 1% Ficoll, 1% polyvinyl pyrollidone in ddH_2O. Aliquoted and stored at -20°C.

DNA Size Markers: Phi(ϕ)X174 RF DNA digested with *Hae*III. Lambda (λ) DNA digested with *Hind*III. Both used at 1μg/10μl.

Ethidium Bromide: 10mg/ml stock in ddH_2O, working concentration 0.5μg/ml. Stored in the dark at room temperature.

Gel Loading Buffer:

1) For DNA gels (10X): 50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol, in 1X TBE. Stored at room temperature.

2) For protein gels (sample buffer): 62.55mM Tris.HCl pH6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue, in ddH_2O. Stored at 4°C.

3) For RNA gels: 50% Formamide, 2.2M Formaldehyde, 1X MOPS, in ddH_2O. Made fresh as required.

MOPS Buffer (10X): 200mM MOPS pH7.0, 50mM potasium acetate, 10mM EDTA. Stored in a light-proof container at 4°C.

Neutralisation Buffer: 1.5M NaCl, 0.5M Tris. HCl pH8.0 in ddH_2O. Stored at room temperature.

Oligonucleotides: Oligonucleotides were synthesised on an Applied Biosystems 3818A Automated DNA Synthesiser (operated by Mr. T. McPherson, Glasgow University, Veterinary Pathology). DNA was removed from columns by using 2ml of ammonia and deprotected by heating overnight at 55°C. Ammonia was removed under vacuum and the resulting DNA pellet was resuspended in TE pH8 at 1µg/µl, and stored at -20°C.

Polyacrylamide Solution With Urea (8%): 39.9g acrylamide, 2.1g bis-acrylamide, 220.5g urea, 52.5ml 10X TBE, in a total of 525ml ddH_2O. Mixed, filtered (0.45µm) and stored in dark glass at 4°C. 50ml of stock solution containing 100µl of 10% ammonium persulphate and 50µl temed was used for each sequencing gel.

Polyacrylamide (30%): Stock solution containing 29.2% acrylamide and 0.8% bis-acrylamide in dark glass and stored at 4°C, supplied by Scotlab.

Phosphate Buffered Saline (PBS): 100mM NaCl, 80mM , 80mM di-sodium hydrogen orthophosphate, 20mM sodium dihydrogen orthophosphate, adjusted to pH7.5. Autoclaved and stored at 4°C.

Pre-Hybridisation Buffer: For nylon membrane (Amersham, Hybond-N): 4X Denhard's, 4X SSC, 50% Dextran sulphate, 25% Formamide, 0.08% SDS, in ddH_2O. Stored at -20°C. 100-300µg/ml of denatured salmon sperm DNA was added before use.

Salmon Sperm DNA: 10mg/ml stock in ddH_2O, denatured by boiling for 10 minutes and cooled slowly on ice. Aliquoted and stored at -20°C.

SSC (20X): 3M NaCl, 0.3M Sodium citrate in ddH_2O. Made to pH7.0 using NaOH, and stored at room temperature.

SSPE (20X): 3.6M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA in ddH_2O. Made to pH8.3 using NaOH.

SOC: 2% Bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl in ddH_2O. Autoclaved and stored at 4°C. Added before use with sterile technique; 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM Glucose.

Tris buffered saline (TBS): 20mM Tris, 137mM NaCl, pH to 7.6 with HCl.

TEA (10X): 400mM Tris.HCl pH8.15, 200mM sodium acetate, 200mM Sodium Chloride, 20mM EDTA. Stored at room temperature.

TE: 10mM Tris.HCl, 1mM EDTA, pH as required. Autoclaved and stored at room temperature.

TBE (10X): 0.9M Tris.HCl, 0.9M Boric acid, 25mM EDTA pH8.3. Stored at room temperature.

Versene (1X): PBS containing 1mM EDTA and 1:100 V:V phenol red. Autoclaved and stored at room temperature.

Western Immunoblot Transfer Buffer (semi-dry electro-blotting): 48mM Tris, 39mM Glycine, 0.01% SDS, 20% MeOH.

X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside) (1000X): 20% W:V in dimethylformamide (DMF). Stored in aliquots protected from light at -20°C.

2.2 Methods

2.2.1 Agarose Gel Electrophoresis

Gels containing from 0.5-1.5% agarose W:V in 1X TBE or 1X TEA were used to separate and analyse DNA molecules (181). 50, 100 or 200ml gels were poured in perspex tanks and wells were cast using appropriate combs. Gel loading buffer was added to samples at a final concentration of 1X, samples were then electrophoresed in 1X TBE/TEA at 5V/cm. When TEA gels were used the buffer was changed frequently to prevent buffer exhaustion. Known concentrations of DNA size markers were run alongside the samples in order to gauge both product size and yield of DNA. Subsequent to running, gels were stained for at least 30 minutes in 1X TBE/TEA with 0.5µg/ml ethidium bromide, then viewed on a short wave UV transilluminator. Photography, when required, was by means of a Polaroid camera.

2.2.1.2 DNA Purification From Agarose Gels

Highly purified DNA was obtained from agarose gels by using the 'Gene Clean' method (Bio 101; supplied by Stratech Scientific Ltd.).

2.2.2 Cell Culture

Feline suspension cells derived from lymphosarcomas (3201, F422, and T17) were grown in plastic flasks (Nunc, Lab Tek), at 37°C in an atmosphere of 5% CO_2. Cells were subcultured every 3 to 4 days and maintained at a density between 5 x 10^5 and 1.5 x 10^6 cells/ml. Growth medium consisted of RPMI-1640 supplemented with 10% foetal calf serum (FCS), 2mM glutamine, 100units/ml penicillin, and 10µg/ml streptomycin (all Gibco, UK).

Primary avian fibroblasts were cultured maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with antibiotics as for RPMI. However, the growth supplements added were 5% newborn calf serum and 1% chick serum plus 10% tryptose phosphate (all GIBCO, UK). Chick serum was heated at 55°C for two hours before use, inactivating any retroviruses present in the serum.

2.2.3 Electrophoretic Mobility Shift Assay (EMSA)

The electrophoretic mobility shift assay is a simple, rapid and sensitive method for detecting sequence-specific DNA binding proteins. This assay can also be used for quantitative determination of the affinity, association rate constants, dissociation rate constants and binding specificity of DNA binding proteins (182). Briefly, an end labelled DNA probe with a consensus binding site is mixed with protein, either crude or fractionated nuclear extract, or as in this case with recombinant protein. The protein/DNA mixture is then subjected to electrophoresis through a non-denaturing polyacrylamide gel, which is then dried and autoradiographed. Discrete bands are detectable where protein-bound DNA complexes are present in the gel.

The electrophoretic mobility shift assay was carried out according to published protocols (182). Reaction volumes were 20µl consisting of xµl protein, 4µl of 5X binding buffer, 2µl ^{32}P probe, 1µl of poly dI/dC @ 1µg/µl, and xµl of water. 5X binding buffer consists of: 100mM Hepes pH 7.2; 250mM KCl; 15mM MgCl; 5mM EDTA; 40% glycerol; 125ng/ml sheared calf thymus DNA. DTT was added to the 5X binding buffer to a final concentration of 1mM just prior to use. Reaction volumes were as stated with protein added at 30ng per reaction for Max and 300ng per reaction for Myc, as the quantity of intact Myc was so low in the eluate.

Reactions were run on a 6% polyacrylamide gel in 1X TBE and electrophoresed at 200 volts. Gels were subsequently dried and exposed to X-ray film (Kodak) overnight, with longer exposures times if required.

2.2.3.4 Annealing and End Labelling of Oligonucleotides Used in EMSA

Oligonucleotides used in the EMSA were double stranded, and therefore the single stranded synthetic oligos had to be annealed. This was achieved by resuspending 10µg of each oligo in a total volume of 200µl of TE buffer, giving 20µg of double stranded

DNA. The DNA was then heated to 95°C, and allowed to cool to room temperature naturally.

End labelling of oligos with ^{32}P was achieved by using T4 polynucleotide kinase, as set out in 2.2.7.2 with 200ng of oligo end labelled per reaction. The 20µl reaction was purified on a 'Nick Column' to remove enzyme and unincorporated radiolabelled ATP.

Sequences of oligos used in EMSA were:-

CM1 sense strand 5'-CCC CCA CCA CGT GGT GCC TGA-3'

P2 sense strand 5'-GAT CCT TGG CAC TCA CGT GGG ACT AGC AG-3'

The consensus Myc/Max binding site is underlined on each oligo.

2.2.4 Hybridisation analysis of DNA, RNA and proteins

2.2.4.1 DNA and RNA hybridisation

Southern Blot Transfer of DNA: This was carried out essentially as described by Southern (183). Briefly, DNA samples were electrophoresed and subsequently the gel was submerged in denaturation buffer for 30 minutes, followed by submersion in neutralisation for 30 minutes. The gel was rinsed in 20X SSC and DNA was then transferred onto Hybond-N nylon membrane by blotting overnight using 20X SSC. The membrane was rinsed briefly with 20X SSC and DNA was crosslinked to the membrane using a UV crosslinker.

Northern Blot Transfer of RNA: This procedure was the same as for the DNA transfer, except that denaturation and neutralisation steps were unnecessary. However, transfer was continued for longer as this improves transfer of larger RNA molecules (181).

Hybridisation Procedure: Standard high stringency conditions for analysis of feline nucleic acids with probes of feline origin were, pre-hybridisation >2hours in 10ml of prehybridisation buffer, radioactive probe was added at $1x10^6$ counts/ml (1-5 ng/ml), hybridise overnight at 42°C. The hybridisation was carried out in bottles constructed for the procedure and placed in an oven designed to turn the bottles (Hybaid, UK). In the morning membranes were rinsed several times in 2X SSC, then given 3 x 20 minute washes in 0.1X SSC/0.5% SDS at 60°C. Membranes were then sealed in polythene, while still moist, and exposed to X-ray film (Kodak).

2.2.4.2 Hybridisation of proteins for western immunoblot analysis

Protein samples were electrophoresed through a denaturing polyacrylamide gel containing SDS and transferred onto PVDF membrane (Immobilon-P, Millipore), using a semi-dry electro blotter (Bio Rad) running at 15 volts for 30 minutes. Before transfer gels were equilibrated in transfer buffer for 10 minutes. After transfer, membranes were blocked for 30 minutes at room temperature in 1X TBS-T (TBS with 0.1% Tween-20), 10% w/v Marvel, and 20% serum derived from the animal which the secondary antibody was raised in. Following blocking the membrane was rinsed and washed 4X for 5 minutes in 1X TBST with 2% w/v Marvel, after which the primary antibody was applied, at an appropriate concentration, in blocking solution with 5% Marvel instead of 10%. The primary antibody was left for 1 hour, with rapid shaking, at room temperature, followed by the same rinsing procedure as above. Secondary antibody was applied following the same protocol as the primary antibody, washes were extended to 4X 10 minutes and Marvel was excluded from the final washes. Finally membranes were developed either by the enhanced chemiluminescence method (Amersham) or by a colourimetric assay. For colourimetric assays, membranes were submerged in substrate buffer (0.1M Tris; 0.1M NaCl; 5mM $MgCl_2$; pH To 9.5) with colour reagent (16mg nitrobluetetrazolium; 8mg 5-bromo-4-chloro-3-indolylphosphate; in 50 ml of substrate

buffer), until colour had developed, then membranes were rinsed with dH_2O to stop the reaction.

2.2.5 Bacterial Transformation

2.2.5.1 Ligation of DNA Molecules

Ligations were carried out according to the manufacturers' protocols supplied with T4 DNA ligase and 5X reaction buffer (GIBCO, BRL, Ltd.). Generally, 50ng of vector DNA and a 3-10 fold molar excess of insert DNA were ligated in a volume of 20µl. Ligations were carried out overnight in a 14°C water bath.

2.2.5.2 Bacterial Transformation

1µl (containing 0.5-5ng of DNA) of a 1/5 dilution of a ligation mix was added to 20µl of competent bacterial cells, which were then incubated on ice for 30 minutes. Cells were subsequently heat shocked at 42°C for 45 seconds and returned to ice for two minutes. 90µl of SOC medium was then added to the cells, which were then incubated, with shaking, for one hour (184). Cells were then plated on to L-agar plates containing ampicillin (50µl ml) and if colour selection was required X-gal was included. These plates were then incubated overnight at 37°C.

2.2.5.3 Identification of Recombinants

For transformation using *lac*Z complementation, white colonies were picked from plates containing X-gal, and grown. When *lac*Z function was already disabled several colonies were picked and grown. In either case picked colonies were grown overnight in 10ml of L-broth at 37°C with shaking and subjected to small-scale plasmid purification.

2.2.5.4 Preparation of Plasmid DNA

2.2.5.4.1 Small Scale Prep

Sequencing grade plasmid was rapidly purified from 1.5ml of overnight culture by using the 'Wizard' miniprep procedure (Promega). Recombinants were identified by restriction digestion of 2-4µl of the purified DNA solution. When recombinants were identified a small amount of the remaining overnight growth was saved by storage in 25% glycerol at -70°C.

2.2.5.4.2 Large scale prep

Bulk preparation of plasmid DNA was by the alkaline lysis procedure given in Maniatis *et al* (181), with final plasmid preparation by centrifugation through CsCl/ethidium bromide gradient.

2.2.6 Polymerase chain reaction (PCR)

The polymerase chain reaction allows the rapid amplification of selected regions of DNA from small amounts of starting template DNA (185). The Perkin Elmer Cetus GeneAmp Kit (supplied by USB, Cambridge Bioscience) was used according to the manufacturer's instructions. This kit utilises a recombinant *Thermus aquaticus* (*Taq*) DNA polymerase which is stable at high temperatures, allowing cyclical high temperature denaturation of template DNA, followed by polymerisation starting from primers targeted to specific regions of the template DNA at lower temperatures. Standard reaction conditions were, depending on the template 10ng to 1µg of template DNA in a 50µl reaction mixture containing 10mM Tris.HCl pH8.3, 50mM KCl, 1.5mM $MgCl_2$, 100µM of each nucleotide and 1µg of each primer and overlaid with 25µl of

mineral oil. A typical cycling reaction was; denaturation at 94°C for 1 minute; annealing at 55°C for 45 seconds; polymerisation at 72°C for 1 minute. Cycling was carried out in 0.5ml reaction tubes in a programmable thermal cycler (Hybaid U.K.). An excellent introduction to this subject can be found in PCR: A Practical Approach (186).

2.2.7 Preparation of radiolabelled DNA fragments

2.2.7.1 Nick Translation

DNA inserts for use as hybridisation probes were gel-purified from their vectors and radioactively labelled using a kit (Amersham), containing the necessary enzymes (DNaseI and DNA polymeraseI) and buffers.

Generally, 100ng of DNA was radiolabelled using 50μCi (1.85 Mbq) of radioactive dCTP in a final volume of 50μl. Unincorporated nucleotides were removed by gel-filtration through Sephadex G50 beads ('Nick Column', Pharmacia), and labelled fragments were eluted in 400μl of TE buffer. The specific activity of the labelled DNA was calculated (usually 1-3 x 10^8 cpm/μg), and the appropriate amount was used immediately as hybridisation probe.

2.2.7.2 End-Labelling of Oligonucleotides

200ng of double-stranded oligonucleotides were kinase labelled in a 20μl volume using 15 units of T4 polynucleotide kinase and 12pmol (γ^{32}P)-ATP, with the appropriate forward reaction buffer. The reaction was incubated for 30 minutes at 37°C, unincorporated label was removed by the 'Nick Column' method.

2.2.8 Sequencing of DNA

DNA sequencing was carried out using a Li-Cor model 4000 DNA sequencer. This type of sequencing relies on reading an infrared label which is incorporated into the DNA during sequencing reactions (187). Very long sequence runs are possible using this machine, with readouts ranging from 500bp up to 900bp.

Sequencing reactions were carried out using a cycle sequencing protocol included with a purpose designed long-read kit, supplied by Epicentre Technologies. Cycle sequencing is based on the chain termination method of Sanger (188), but uses a thermostable DNA polymerase to give multiple rounds of high temperature DNA synthesis. Briefly, a 17µl reaction consisting of 500ng to 1µg of plasmid DNA, 2pmol of IR-labelled primer, 2.5µl 10 x sequencing buffer, 1µl SequiTherm thermostable DNA polymerase, and ddH_2O to17µl, was split into 4 x 4µl aliquots in 0.5ml PCR tubes and 2µl of the appropriate termination mix was added. Subsequently 10-15µl of mineral oil was overlaid on the reaction, and reactions were denatured for 5 minutes at 95°C then passed through 30 cycles with 95°C for 30 seconds, 60°C for 30 seconds, and 70°C for 1 minute. After cycling, 4µl of stop solution was added to each reaction and the reactions were stored on ice in the dark before use. It was found that reactions remained viable for up to two weeks after cycling, if the reactions were stored at -20°C.

Commonly used IR-labelled primers are shown below.

<u>Universal primers</u>

M13(-29) forward primer 5'-CAC GAC GTT GTA AAA CGA C-3'

Reverse primer 5'-GGA TAA CAA TTT CAC ACA GG-3'

<u>GST-fusion construct primers</u>

5' pGex primer 5'-GGG CTG GCA AGC CAC GTT TGG TG-3'

3' pGex primer 5'-CCG GGA GCT GCA TGT GTC AGA GG-3'

2.2.9 Primary Chick Cell Assays

2.2.9.1 Production of pSFCV-LE sa$^+$/*myc* Vectors

To facilitate cloning of the c-, and v-*myc* genes into the replication defective pSFCV-LE sa$^+$ vector (174), polymerase chain reactions were carried out, using primer sequences which included restriction enzyme sites to allow directional cloning of the reaction products into the vector. Amplification of full length *myc* sequences used the plasmid clones T17M (189) and pBam8 (190) as templates. Primer sequences were as follows:-

c-*myc* forward primer 5'-GCG ACA AGC TTG GAA AAC CCG CAG GCT GCC-3'

c-*myc* reverse primer 5'-GCG ACG AAT TCC CAG TTC CTC CCT CTA ATA GG-3'

v-*myc* forward primer 5'-GCG ACA AGC TTC AAG AAG AGA TCC AGA GAC-3'

v-*myc* reverse primer 5'-GCG ACG AAT TCC AGA GCC CTC CCT CTA ATA GG-3'

Forward primer sequences were designed to hybridise upstream of the ATG start site, while the reverse primers were directed to sites downstream of the TAA stop codon. Restriction sites are underlined and correspond to HindIII and EcoRI on the forward and reverse primers respectively. PCR reactions were as previously described in 2.2.6. PCR products were initially cloned into the pCRII vector and sequenced before subsequent subcloning into the pSFCV-LE sa$^+$ vector. Apart from coding for the *neo* gene, the

vector contains a splice acceptor site upstream of the cloning site, allowing expression of the inserted genes from subgenomic mRNAs. Once the *myc* genes were subcloned into the pSFCV-LE vectors large scale plasmid preparations were prepared by alkaline lysis followed by caesium chloride density centrifugation.

2.2.9.2 Construction of pSFCV-LE:c/v- and v/c-*myc* Chimaeras

Rather than construct completely new vectors for this set of experiments, the original pSFCV-LE/*myc* vectors were modified. This was achieved by digestion of 1μg of each vector with the restriction enzymes HindIII and BfrI (an isoschisomer of Afl II). HindIII cuts at the original 5' cloning site, while BfrI cuts at 889bp after the "A" of ATG in c-*myc* and 667bp after the "A" of ATG in T17-*myc*. The restriction digests were electrophoresed on a 1.2% TAE agarose gel beside undigested plasmids, after staining with ethidium bromide and visualisation with UV, the various fragments were excised from the gel using sterile scalpels. DNA fragments were recovered from the agarose slices by use of the "Gene Clean" method. Subsequent quantification of recovered DNA was estimated by comparison with known standards, after running an aliquot on a 1.2% TAE agarose gel , and visualisation as before. Digested and purified N-terminal DNA fragments were then ligated into the appropriate digested vectors, and ligations were used to transform DH5α bacteria. Bacterial colonies were tested by plasmid mini prep of DNA and double restriction digestion with HindIII and EcoRI, followed by gel electrophoresis of the digests. One bacterial colony containing each chimaera was grown as a bulk preparation of plasmid DNA, and plasmid was prepared by caesium chloride density centrifugation (Section 2.2.5.4.2). To confirm that plasmids contained the expected chimaeric construct, each recombinant plasmid was partially sequenced.

2.2.9.3 Transfection and Drug Selection of Primary Chick Cells

Primary chick cells were plated at a density of $2x10^5$ cells per $25cm^2$ tissue culture flask and cultured as described in section 2.2.2. Transfection was by the calcium phosphate method (191,192) using a kit supplied by Stratagene. 10μg of each construct was transfected along with 4μg of the replication competent avian retroviral vector RCAN (193), thus allowing limited viral spread. After transfection, cells were grown to confluence and then split 1:2 into fresh medium containing 1mg/ml G418. Cells were kept in this initial selection for 8 to 10 days after which time non-transfected control cells had died and colonies of drug resistant cells had begun to grow up in the transfected flasks. Drug resistant cells were then used to assay the effects of the *myc* constructs on growth rate and anchorage independence parameters, as well as apoptosis under low serum conditions.

2.2.9.4 Growth Rate Assay

Cells were plated at a density of 10^5 per 35mm diameter tissue culture dish and grown for 24,48,72, or 96 hours. Three dishes of each type of transfected cells were used for each time point, with a mean count calculated from the three dishes. After the requisite time interval the dishes were washed twice in versene and the cells were removed using 1ml of trypsin/versene, each dish of cells was then counted using a haemocytometer.

2.2.9.5 Growth in Soft Agar

Drug selected cells were assayed for their ability to grow in a semi-solid medium consisting of a base layer of 0.6% agar with cells suspended at $2.5x10^4$ or $5x10^4$ cells

per ml of 0.3% agar, in a 75mm diameter tissue culture dish. Cells were then incubated in a humidified incubator and fed with 1ml of complete medium once per week.

2.2.9.6 Western Blot Detection of Myc in Chick Embryo Fibroblasts

Although cells used in the experiments had been subjected to drug selection, western blot immunodetection was undertaken to confirm that the cells were expressing Myc proteins of the expected size. The protocol for this was as previously described in 2.2.4.2 and 3.2.4, using a 12.5% SDS-PAGE gel.

2.2.9.7 Apoptosis Analysis by Terminal Transferase labelling and FACS Counting

For a quantitative measure of cells which entered apoptosis, upon serum deprivation, cells were labelled with biotin-16-dUTP which is incorporated into single stranded DNA ends by the enzyme terminal deoxyribonucleotidyl transferase (TdT). Cells which have multiple strand breaks can then be visualised by the addition of avidin-FITC, as the avidin binds to biotin and the fluorescent label FITC can be detected by laser excitation at the correct wavelength (194).

Transfected cells were split and grown until between 30% and 50% confluent, as high densities can inhibit Myc-induced apoptosis (279), at which point all cells were rinsed with serum free medium, and cells were then incubated in media in the presence (controls) or absence of serum. These cells were then grown for 15 hours under normal growth conditions. The assay was carried out by removing and storing the media from all flasks and combining with cells which were still adherent at the end of the 15 hour time period. Each batch of cells was fixed by suspension in 1% paraformaldehyde/PBS pH7.4 for 15 minutes at 4°C, followed by washing with PBS. The cells were then resuspended in 50µl of TdT staining buffer (0.1M Na cacodylate pH7, 1mM $CoCl_2$, 0.1mM DTT, 0.05mg/ml BSA), plus 0.5nmoles of biotin-16-dUTP and 10 units

terminal transferase, per reaction. Reactions were then incubated for 1 hour at 37°C. After incubation cells were washed in PBS then resuspended in 100µl of staining buffer (4 x SSC, 0.1% Triton-X, 5% w/v marvel and 2.5 µg/ml FITC-avididn), and incubated for 30 minutes at room temperature, in the dark. Finally, cells were washed in PBS/0.1% Triton-X, before being subjected to analysis on a Coulter Epics ELITE flow cytometer.

2.2.10 GST-fusion proteins

2.2.10.1 Max Expression as a GST Fusion Protein

The feline *max* gene was amplified by the PCR using primers which included a BamHI restriction site in the 5' primer and an EcoRI restriction site in the 3' primer. The restriction sites allow directional in-frame cloning of the PCR fragments into the pGex 2T vector (175), after restriction digestion of gel purified PCR fragments. Oligonucleotide sequences are as follows:-

5' Primer 5'-GCA GGA TCC ATG AGC GAT AAC GAT GAC ATC G-3'

3' Primer 5'-GCA GAA TTC GGA ATT CGG CTT GGC TTA GCT G-3'

The BamHI and EcoRI restriction sites are underlined on the 5' and 3' primers respectively.

After ligation into the pGex2T vector and transformation of DH5α bacteria, colonies of bacteria which contained plasmid with insert, as shown by plasmid mini-prep followed by restriction digestion with BamHI and EcoRI and gel electrophoresis, were assayed for their ability to express the heterologous protein. Initially small scale protein prep analysis were carried out by inoculating 10ml of L-broth with the relevant colonies, these were grown overnight with aeration in a shaking incubator at 37°C. The next morning 2ml of fresh L-broth was inoculated with 200µl of the overnight growth, this

was grown for another 2.5 hours under the same conditions as the overnight culture. After this further period of growth, cultures were induced to produce the heterologous protein by addition of 0.1 mmol/l isopropyl β-D thiogalactoside (IPTG) and grown for a further 2 hours. Following induction 1.5ml of each culture was pelleted in a 1.5ml eppendorf tube, and resuspended in 200µl of sample buffer, this was then boiled for 5 minutes and an aliquot was run on a 10% SDS-PAGE denaturing gel alongside a similar aliquot from uninduced bacteria.

When productive colonies were identified, large scale protein preparations were carried out as described in (175,195). Briefly, 100ml of L-broth was grown overnight with bacteria containing the pGex-Max construct, then diluted 1:10 with L-broth supplemented with 5g/l glucose and grown until the A_{600} was between 0.5 and 2.0. Protein induction was then initiated by addition of IPTG to a final concentration of 0.1mmol/l. The culture was then grown for a further 2 hours after which the bacteria were harvested by centrifugation at 8000g for 15 minutes. Bacterial pellets were resuspended in 0.01 x initial volume of Tris buffered saline (TBS) containing protease inhibitors (20 mmol/l benzamidine, 0.1 mmol/l phenyl methyl sulphonyl fluoride (PMSF) and 50 mmol/l EDTA). The resulting suspension was then sonicated on ice for three 15 second periods using a 100w ultrasonic disintegrator (Fisons, Crawley, England) and the lysed bacterial preparation was centrifuged at 1800g for 30 minutes and the supernatant retained. At this stage it was possible to store the supernatant overnight at -20°C without significant loss of fusion protein. The supernatant was then incubated at room temperature with pre-swollen glutathione-agarose beads, with gentle agitation for 20 minutes. This slurry was then spun down and washed repeatedly with TBS until the optical density at 280nm had returned to that of TBS. Recombinant protein was then eluted from the beads by washing with TBS containing 10mmol/l reduced glutathione. Yield of recombinant protein was estimated by the method of Bradford (1865) and/or optical density at 280 nm, with O.D. 1 = 0.5 mg/ml of protein. Purified protein was subjected to SDS-PAGE denaturing electrophoresis on a 10% gel, followed by

Coomassie blue staining. As can be seen from figure 6.2 GST-Max is a relatively stable construct.

2.2.10.2 Expression of Recombinant GST-Myc BR-HLH-LZ

For the GST-Myc constructs the C-terminal 115 amino acids of c-Myc and 116 amino acids of T17-Myc were cloned in-frame into pGex 2T. These were then expressed and purified as in 6.2.2. Again DNA inserts was generated by the PCR, primer sequences were :-

5'-GCG ACG GAT CCG CTA AGT TGG ACA GTG GCA GG-3'

5'-GCG ACG AAT TCC CAG TTC CTC CCT CTA ATA GG-3'

C-*myc* 5' and 3' primers with BamHI and EcoRI restriction sites underlined,

5'-GCG ACG GAT CCG CTA AGT TGG GCA GTG GCA GG-3'

5'-GCG ACG AAT TCC AGA GCC CTC CCT CTA ATA GG-3'

T17-*myc* 5' and 3' primers, again restriction sites are underlined, also shown in bold type are changes in the T17 sequence. Purified recombinant protein was run on a 10% SDS-PAGE denaturing gel, however the GST-Myc proteins proved to be unstable as can be seen from figure 6.1.

2.2.10.3 Construction of GST-Myc N-terminal Proteins

Myc N-terminal fusion proteins were constructed by the PCR using primers which encode a BamHI site in the 5' primer and an EcoR1 site in the 3' primer. PCR reactions were carried out as described in materials and methods. Primer sequences were:-

5' primer 5'-GAC GGA TCC ATG CCC CTC AAC GTC AGC-3'

3' primer 5'-GAC GAA TTC TCT TCC TCA GAG TCG CTG-3'

These gave fusion products consisting of 254 amino acids of c-Myc and 180 amino acids of T17-Myc.

2.2.10.4 Western Immunoblot of GST-Myc Fusion Proteins

To ensure that the GST-Myc proteins were being correctly expressed, western immunoblot analysis was carried out on purified proteins. The antibody used to detect Myc was the rabbit polyclonal Ab2736 used at 1:1000, and the secondary antibody was a mouse anti-rabbit monoclonal conjugated to alkaline phosphatase, used at 1:3000 (Promega Cat. No. 53731).

CHAPTER 3

Chapter 3 Analysis of transduced *myc* genes in secondary tumours induced by the T17 virus complex

Chapter 3 Analysis of transduced *myc* genes in secondary tumours induced by the T17 virus complex

3.1 Introduction

The T17 *myc* mutant was discovered as a gene transduced by FeLV, in a field case of feline lymphomasarcoma. Also found in the same cancer was a FeLV transduced T-cell receptor β-chain gene (173). The *myc* containing provirus was cloned from an EcoRI-digested DNA library of T17 tumour DNA in λEMBL4 and subcloned into pUC18 (189). Figure 3.1 shows the clone T17M containing the entire provirus of approximately 7.5kb, together with cellular sequences flanking the provirus. Sequencing of the provirus showed that the transduced *myc* gene accounted for 1146 base pairs, replacing bases 5,719 - 7,568 of FeLV. Figure 3.1 also shows the open reading frames of the transduced cellular insert.

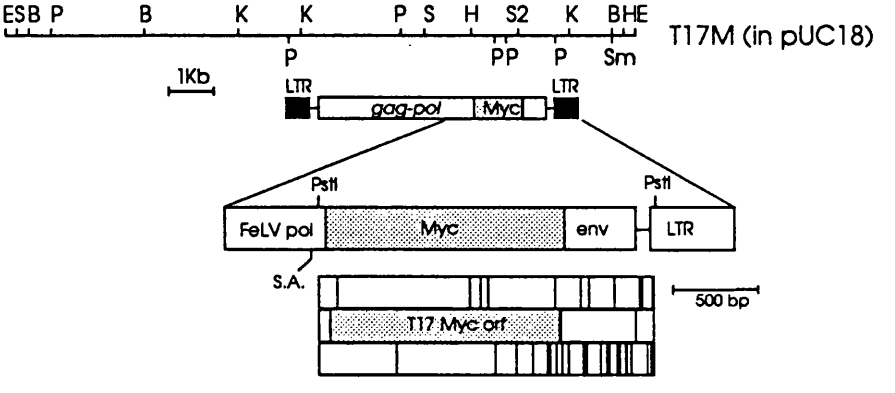


Figure 3.1

Figure 3.1 Map of the T17 *myc* clone.

Vertical lines in the lower part of this figure denote 'stop' in the three possible reading frames.

The sequence data leads to a predicted T17 *myc* product which has a -1 shift relative to the FeLV *pol* reading frame which terminates in the transduced gene. The ATG of T17 v-*myc* is 68 base pairs downstream of the consensus splice acceptor for the FeLV *env* gene, suggesting that expression of the T17 Myc protein will be as a discrete product from a subgenomic spliced mRNA. As only 3 base pairs from exon one of the cellular gene remain, the T17 mutant does not encode the higher molecular weight exon-1 initiated gene product (196), while previous work using an exon 1 probe had shown that no c-*myc* RNA was detectable in these cells (18).

Sequence analysis also gave the rather unexpected finding that the coding sequence contained a large bipartite deletion in the N-terminal coding region. These deletions of 141 and 81 base pairs flank 8 base pairs which differ from c-*myc* by a single base change. The whole effect is to delete 74 amino acids and introduce two codon changes, while retaining the correct reading frame for the rest of *myc*. Further mutations are found in the C-terminal coding region with an A to G transition at position 983 (numbering from A of ATG in c-*myc*) changing asp328 to glycine. More intriguing is a three base insertion (TCG) which converts leu362 to phenylalanine-arginine, disturbing the conserved helical configuration of the sequence specific DNA binding domain which interacts with the consensus CACGTG motif (114), this domain is the basic region (BR).

Infection of cats with supernatant from a cell line derived from the original T17 tumour, resulted in tumours being generated in the newly infected animals. The genesis of these new tumours had a time scale only marginally slower than tumours which are generated from infection with other FeLV isolates containing full-length tranduced *myc* gene (197). However, it was possible that the rapid onset of tumours in these animals was due to the action of the replication competent component of the T17 virus complex, and/or the *tcr*-containing virus. To ascertain whether the T17 mutant was merely an aberration thrown up in the original field case, or whether it is of biological significance, the secondary tumours were subjected to an in-depth analysis.

The first step was to examine the secondary tumours by Southern blot, to ask whether the *myc* containing virus was present in the secondary tumours. This question was further extended to ask whether any transduced *myc* allele was of the original highly mutated form including the gross deletions and the mutated basic region. The latter question was approached by using a polymerase chain reaction method, followed by sequence analysis. Previous work had shown that v-*myc* mRNA was detectable in T17 tumour cells using a *myc* probe, while a first intron probe failed to detect any c-*myc* mRNA (18). To discover whether a truncated protein was expressed western blot analysis was undertaken.

3.2 Methods

3.2.1 Restriction digestion of tumour DNA and Southern blot analysis

DNA had previously been isolated from the tumours J49/1, J49/2 and J53/2, by Mrs. A. Terry (197), and it was this material which was used for analysis. Digests of 20μg total DNA, were carried out using the restriction enzyme HindIII in suitable volumes. Digests were reduced in volume to approximately 50μl and electrophoresed on a 0.8% TAE agarose gel. The procedure for transfer onto the nylon membrane was as previously described (Section 2.2.4.1).

3.2.2 Polymerase chain reaction analysis of tumours

Polymerase chain reactions were carried out on tumour DNA with primers designed to anneal to the 5' end of *myc*, while a 3' primer was designed to hybridise with a sequence present in the *env* gene of FeLV. Thus polymerisation of nucleotides would only occur

if a FeLV provirus was present and contained a transduced *myc* gene The 3' *env* primer enabled the detection of possibly any transduced *myc* as the primer sequence is just downstream of an FeLV *env* sequence which acts as a splice acceptor for *myc* (198). Primer sequences are shown below.

5' primer (*myc*)	5'-ATG CCC CTC AAC GTC AGC-3'
3' primer (*env*)	5'-CGG TGT GAT CCG CAT AGA AGC-3'

These primers were used in reactions which contained 100pmol of each primer with 1μg of total DNA derived from the tumours, each reaction was supplemented with 10% DMSO. The DMSO was required only for PCR of the N-terminal portion of *myc* which is rich in G and C nucleotides, and may act by inhibiting the forming of secondary structures which stop the polymerase from making full-length nucleotide chains.

3.2.3 Cloning and sequencing of PCR products

Blunt end cloning of PCR products was problematic, until it was realised that the thermostable polymerase added an extra adenine residue to the 3' end of nucleotide chains which were synthesized. This problem has been overcome by the use of cloning vectors which carry a 3' thymidine residue overhang, hence the use for this work of the pCRII vector which has proved to be very efficient in cloning PCR fragments. A further advantage is that the vector contains sites for the M13 forward and reverse sequencing primers. Thus, sequencing of cloned products was carried out directly on colonies carrying the correct sized insert as demonstrated by small scale plasmid purification, followed by restriction digest with EcoRI and gel electrophoresis. Sequence data was generated by use of the aforementioned M13 forward and reverse primers, however these primers were labelled with an infra red dye and sequencing was performed using a LiCor 4000 automated sequencer (187). In order to ensure reliable sequence data internal IR-labelled primers were also the sequences of these primers being:-

Forward	5'-GGA CTC TCT GCT CTC CTC-3'
Reverse	5'-GAG GAG AGC AGA GAG TCC-3'

3.2.4 Western blot analysis of the T17 cell line

Cultured thymocyte cell lines were harvested at the appropriate cell numbers and washed with PBS. Cells were then lysed in sample buffer containing the protease inhibitors leupeptin, aprotinin and PMSF. The cell lysates were then electrophoresed on a 12.5% SDS-PAGE gel and hybridised as previously described (Section 2.2.4.2) to PVDF membrane. The blot was probed using a monoclonal antibody raised against the carboxy terminus of c-Myc at a dilution of 1:1000 (gift of Dr. K. Moelling, Lausanne). Proteins were subsequently visualised by the enhanced chemiluminescence method.

3.3 Results

3.3.1 Hybridisation analysis of secondary tumours induced by the T17 virus complex

Southern blot hybridisation analysis confirmed that there were novel *myc* alleles present in the tumours J49/1, J49/2 and J53/2. As can be seen from figure 3.2 each of the tumours contains a transduced *myc* gene, provides evidence that virus in the supernatant of T17 cells can transmit a *myc* gene with oncogenic properties. To iterate, we might expect that a defective feline leukaemia virus, which requires a helper virus, would be lost if the transduced gene had no role to play in the genesis of a tumour, as there should be no selection pressure to retain a defective virus.

If proviral integration itself was a major factor in the tumour initiation, which has been noted in several different systems, we would expect that a particular genetic locus would be targeted by the provirus (199). What is clear from this Southern blot is that the provirus has integrated at a different locus in each of the host genomes, making it

unlikely that the tumours have arisen as a result of proviral integration leading to deregulation of a particular cellular gene, and allied to other cooperating event(s).

3.3.2 PCR of secondary tumours

From Figure 3.3 the size of the transduced *myc* genes can be determined and shown to be in the same size range as the original T17 *myc*, that is at ~1200bp. Tumours J49/1, J49/2 and J53/2, also demonstrate that no other larger band is present, which is in the size range of the F422 cell line derived PCR band this being equivalent to a full length transduced *myc* allele. Thus, the PCR reaction products demonstrate that no recombination has occurred between v-*myc* and c-*myc* to repair the deletions of the transduced *myc* genes in any of these tumours.

3.3.3 Sequences of *myc* in secondary tumours

To ascertain whether those PCR products which were generated contained the same mutations as the original mutant, clones of each product were sequenced. Results of the sequencing confirmed that the gross mutations were indeed present, in the same form as the original transduced gene. An alignment of the mutant sequences with c-*myc* is given (Figures 3.5 and 3.6) which shows in detail where the mutations occur.

3.3.4 Western blot protein detection in T17 cell line

Detection of the truncated protein in figure 3.4 confirms that the transduced mutant is expressed as a subgenomic RNA which is efficiently translated into the predicted size of polypeptide. The seemingly large quantity of protein product is more likely to be a due

to the stability of the protein, than any inherent ability of the virus to transcribe greater numbers of genomic viral RNA copies than other *myc* containing viruses, although other possibilities, such as more stable mRNA, cannot be excluded as the explanation for the excess of the mutant protein.

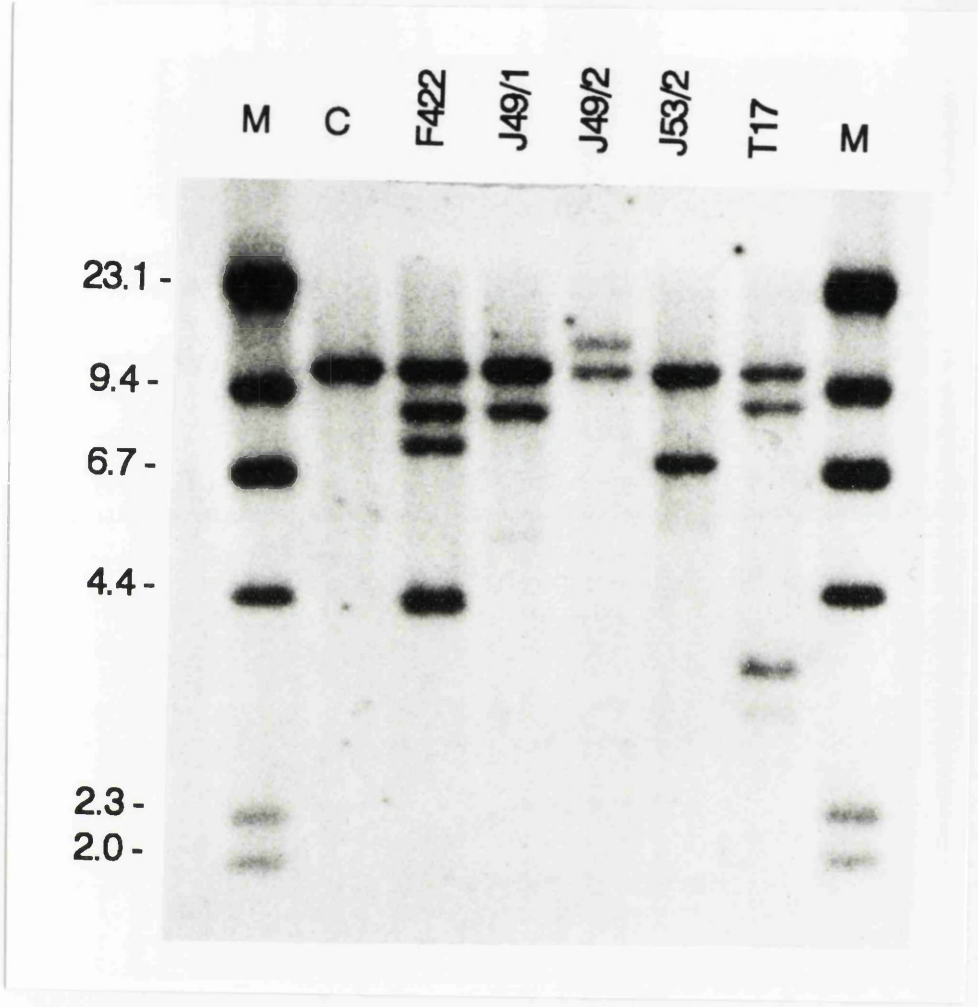


Figure 3.2

Southern blot analysis of secondary tumours induced by the T17 virus complex.

M, markers (λ); C, control DNA; F422, DNA from cell line containing a full length v-*myc*; J49/1, J49/2, J53/2, secondary tumour DNA; T17, DNA from the original lymphoma derived cell line.

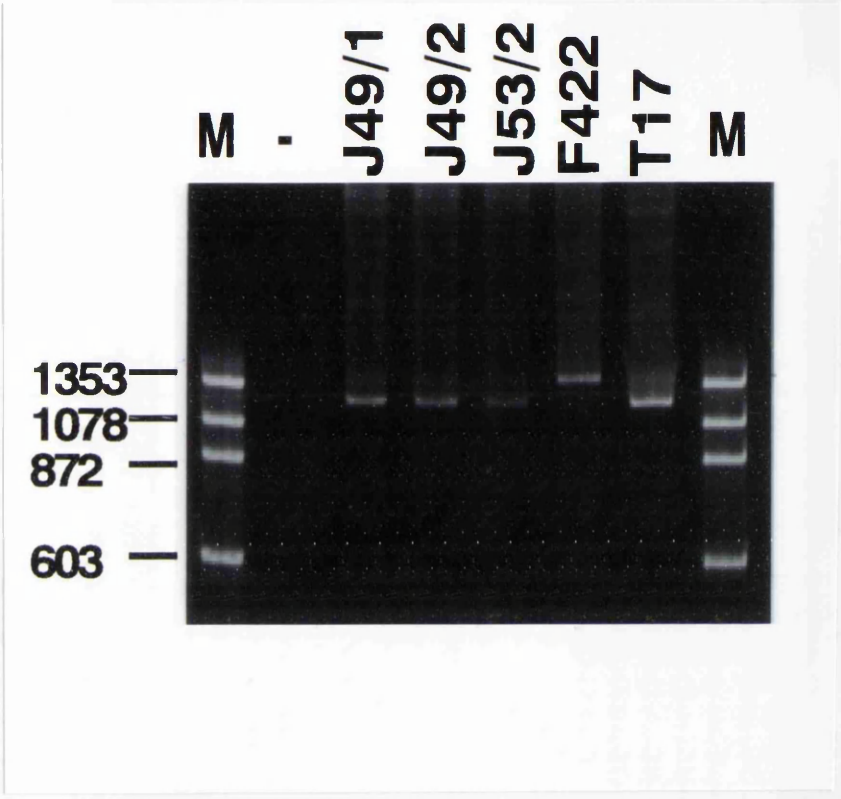


Figure 3.3

Polymerase chain reaction analysis of tumour DNA from secondary tumours.

M, markers (ϕ); -, negative control reaction; J49/1, J49/2, J53/2, secondary tumour DNA reactions; F422, full length transduced v-*myc*; T17, original lymphoma derived T17 v-*myc* DNA.

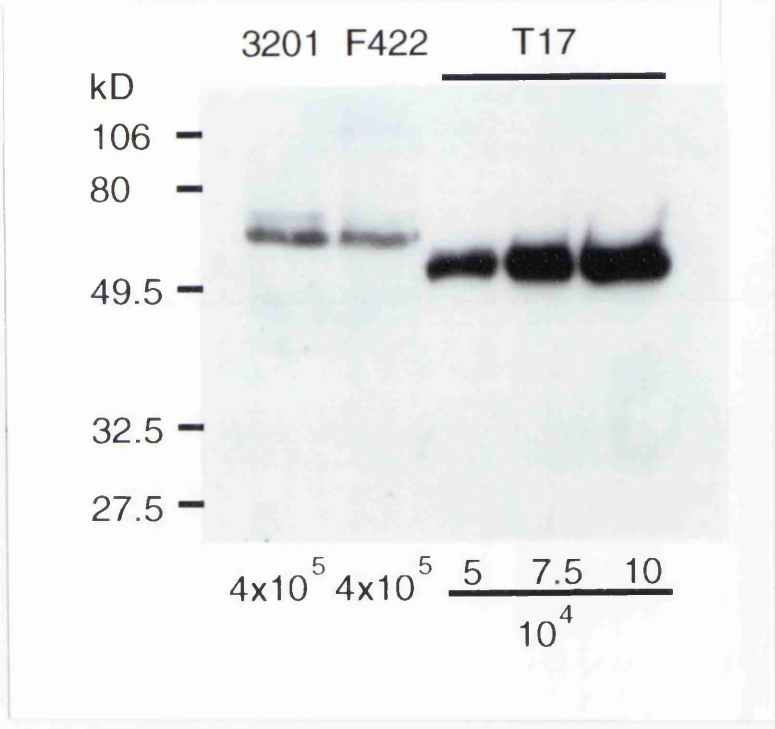


Figure 3.4

Western immunoblot of Myc expressing cell lines. Cell numbers are given beneath each lane.

The 3201 cell line also shows the slower mobility P1 promoter derived protein moiety.

3.4 Discussion

The results in this chapter support the hypothesis that the T17 *myc* mutant does have oncogenic potential: firstly, by the finding that a virus containing a transduced a *myc* gene has integrated into the genome of tumour tissue of infected cats; secondly, sequence analysis of these secondary tumours reveals that mutations present in the original T17 *myc* mutant have been retained in the secondary tumours; thirdly, the protein product of the transduced allele is present at extremely high concentrations, which exceed by greater than one order of magnitude those concentrations found in cell lines which contain either a full-length transduced allele, or have a germ line rearrangement of *myc*.

This evidence strongly supports the hypothesis that the *myc* containing virus has oncogenic potential, rather than a model in which tumours arise as a stochastic event due to proviral integration. If the latter case were true then one might expect that the virus containing the transduced T-cell receptor (TCR) would integrate with the same frequency into cells. However, this does not appear to happen as no trace of the TCR was found in the secondary tumours, although it was shown that the TCR-containing virus was present in the inoculum (197). Also germane is the fact that the virus inoculum contains competent helper virus. Therefore, any defective virus is likely to be selected against as it requires replication competent helper virus to propagate, unless there is selective pressure to maintain the defective virus due to some advantage conferred on the infected cells, e.g. an inability to leave the cell cycle, or preventing cells from entering the differentiation programme.

The idea of a selective advantage being conferred by the mutant is supported by previously published work with transformation defective v-*myc* genes. In these *in vivo* studies deletion mutants of the avian MC29 *myc* containing virus which were partially transformation defective, were rapidly repaired by recombination with c-*myc* to yield

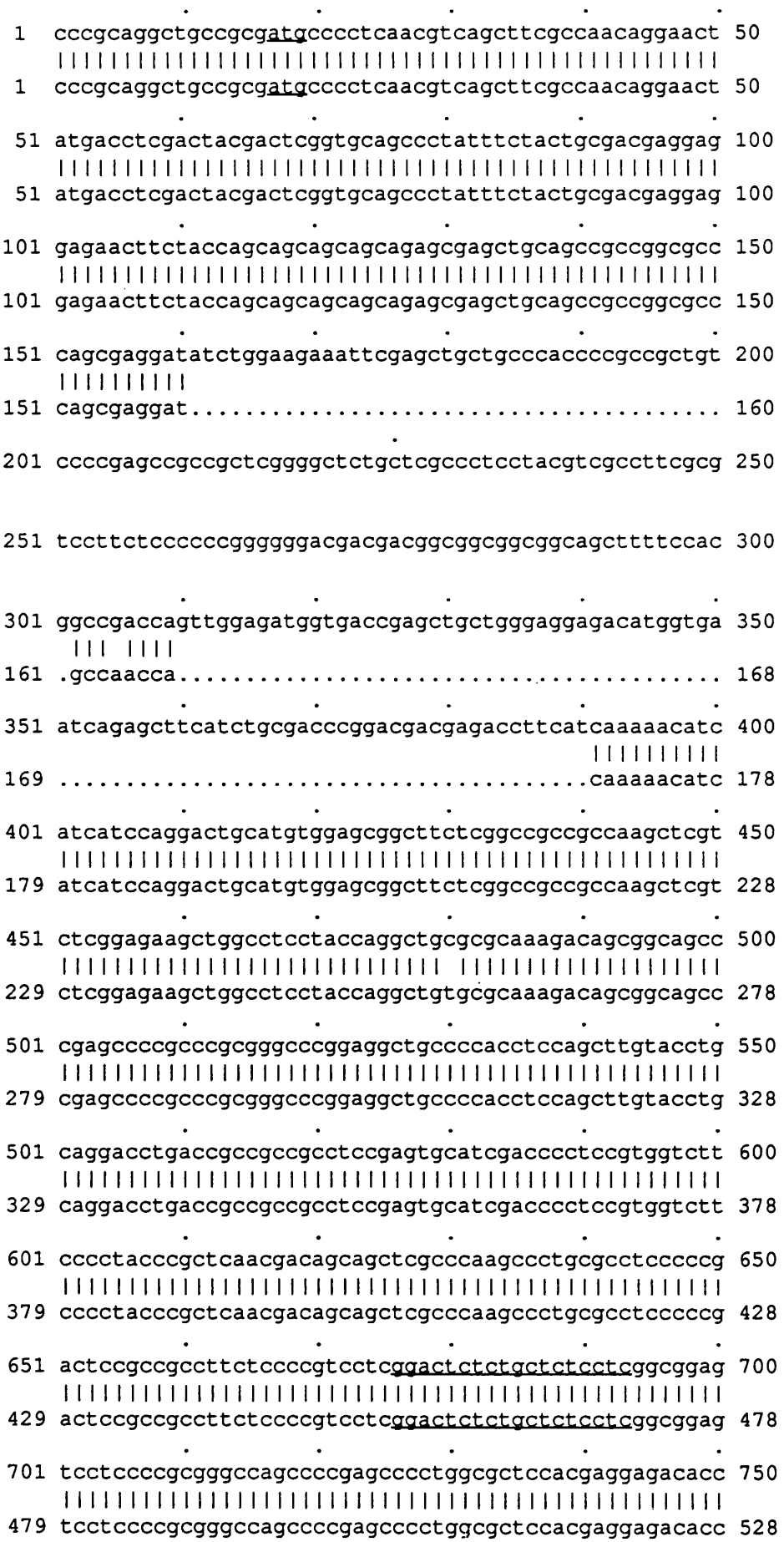
fully transforming genes (200). The hypothesis that a deficient viral oncogene would be repaired is further supported by work on transformation defective avian sarcoma virus. In this system it was found that virus containing v-*src* mutants induced tumours after a short delay, and examination of the v-*src* again showed that recombination event(s) had occurred with the cellular equivalent, restoring the full length gene sequence (201). (Intriguingly the previous two examples suggest that v-*onc* genes might undergo recombination with cellular equivalents, at a relatively high frequency.) Thus we would expect that any deficiency in the T17 mutant would be repaired by recombination, leading to full length transduced *myc* sequences being present in the secondary tumours.

However, the most striking feature of the T17 *myc* mutant is the large bipartite in-frame deletion in the N-terminal coding sequence. Previous work using N-terminal deletion mutants all concluded that this region is important for fibroblast transformation. Examples of this include the study of Heaney (202), while Stone *et al* showed that deletion of amino acids 105-143 appeared to be essential for *myc-ras* co-transformation of rat embryo fibroblasts (REF), and other deletion mutants including D41-53, D55-92 and D93-103 also had a significant effect (203). This assay also revealed that amino acids 3-38 contain a region important for activity (ibid.) and this is intact in the T17 mutant.

The very high levels of T17 Myc protein are without precedent. This level of Myc protein is most likely explained by an increase in the stability of the protein. Myc protein normally has a short half life of 15-20 minutes in cells (11), however 24 of the 74 deleted amino acid residues have been postulated to be important markers of proteins with a short half life, this is known as the PEST hypothesis (204), an acronym utilising the single letter code of the amino acids involved, viz. proline, glutamic acid, serine and threonine, which are abundant in domains of many proteins with a short half life. Also included in these deleted sequences are two phosphorylation sites which can modulate Myc function, by either potentiating (205,206) or suppressing (207) Myc transformation

and transactivation. However, these seemingly irreconcilable results may be due to the systems employed, or due to the nearby phosphorylation sites (Thr 58 and Ser 62) having differing functions, as mutation of Thr 58 to alanine was able to increase focus formation, while mutation of Ser 62 decreased transformation in the same assay (208).

With the findings detailed in this chapter it was decided to assay the effects of the T17 *myc* mutant in an established *myc* transformation assay.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301 ggccgaccagttggagatggtgaccgagctgctgggaggagacatggtga 350
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169 caaaaacatc 178
401 atcatccaggactgcatgtggagcggcttctcggccgccgccaagctcgt 450
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451 ctcggagaagctggcctcctaccaggctgcgcgcaaagacagcggcagcc 500
229 ctcggagaagctggcctcctaccaggctgtgcgcaaagacagcggcagcc 278
501 cgagccccgcccgcgggcccggaggctgccccacctccagcttgtacctg 550
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429 actccgccgccttctccccgtcctcggactctctgctctcctcggcggag 478
701 tcctccccgcgggccagccccgagcccctggcgctccacgaggagacacc 750
479 tcctccccgcgggccagccccgagcccctggcgctccacgaggagacacc 528

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751 gcccaccaccagcagcgactctgaggaagaacaagaggaagaagaagaaa 800
    ||||||||||||||||||||||||||||||||||||||||||||||||||
529 gcccaccaccagcagcgactctgaggaagaacaagaggaagaagaagaaa 578

801 ttgatgtcgtttctgtggagaaaaggcagccccctgccaaaaggtcggaa 850
    ||||||||||||||||||||||||||||||||||||||||||||||||||
579 ttgatgtcgtttctgtggagaaaaggcagccccctgccaaaaggtcggaa 628

851 tcggggtcaccctctgccggaggccacagcaaacctcctcacagcccgct 900
    ||||||||||||||||||||||||||||||||||||||||||||||||||
629 tcggggtcaccctctgccggaggccacagcaaacctcctcacagcccgct 678

901 ggtccttaagagatgccacgtgcccacccaccagcacaattacgcagcgc 950
    ||||||||||||||||||||||||||||||||||||||||||||||||||
679 ggtccttaagagatgccacgtgcccacccaccagcacaattacgcagcgc 728

951 ccccctccactaggaaggactacccagccgccaagagggctaagttggac 1000
    |||||||||||||||||||||||||||||||||||||||||||||||| |
729 ccccctccactaggaaggactacccagccgccaagagggctaagttgggc 778

1001 agtggcagggtcctgaaacagatcagcaacaaccgcaaatgtatcagccc 1050
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 779 agtggcagggtcctgaaacagatcagcaacaaccgcaaatgtatcagccc 828

1051 caggtcttcggacacggaggagaacgacaagaggcggacgcacaacgtct 1100
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 829 caggtcttcggacacggaggagaacgacaagaggcggacgcacaacgtct 878

1101 t...ggaacgccagaggagaaacgagctgaaacggagctttttgccctg 1147
     |   ||||||||||||||||||||||||||||||||||||||||||||||
 879 ttcgggaacgccagaggagaaacgagctgaaacggagctttttgccctg 928

1148 cgcgaccagatcccagagttggaaaacaacgaaaaggcccccaaggtggt 1197
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 929 cgcgaccagatcccagagttggaaaacaacgaaaaggcccccaaggtggt 978

1198 gatccttaaaaaggccaccgcgtacatcctgtccgtccaagcaggggagc 1247
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 979 gatccttaaaaaggccaccgcgtacatcctgtccgtccaagcaggggagc 1028

1248 aaaagctcatttcggaaaaggacctgttgaggaagcgacgagaacagttg 1297
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1029 aaaagctcatttcggaaaaggacctgttgaggaagcgacgagaacagttg 1078

1298 aaacacaaacttgaacagctaaggaactcttgtgcataagtccacctatt 1347
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1079 aaacacaaacttgaacagctaaggaactcttgtgcataagtccacctatt 1128

1348 agagggagg 1356
     |||||||||
1129 agagggagg 1137
```

Figure 3.5

Alignment of T17 v-*myc* (bottom) with c-*myc* (198). The ATG start site and the TAA stop of the open reading frames are underlined. Also underlined is the region where

internal forward and reverse IR-labelled primers were designed to anneal to. Numbering of the sequence is arbitrary.

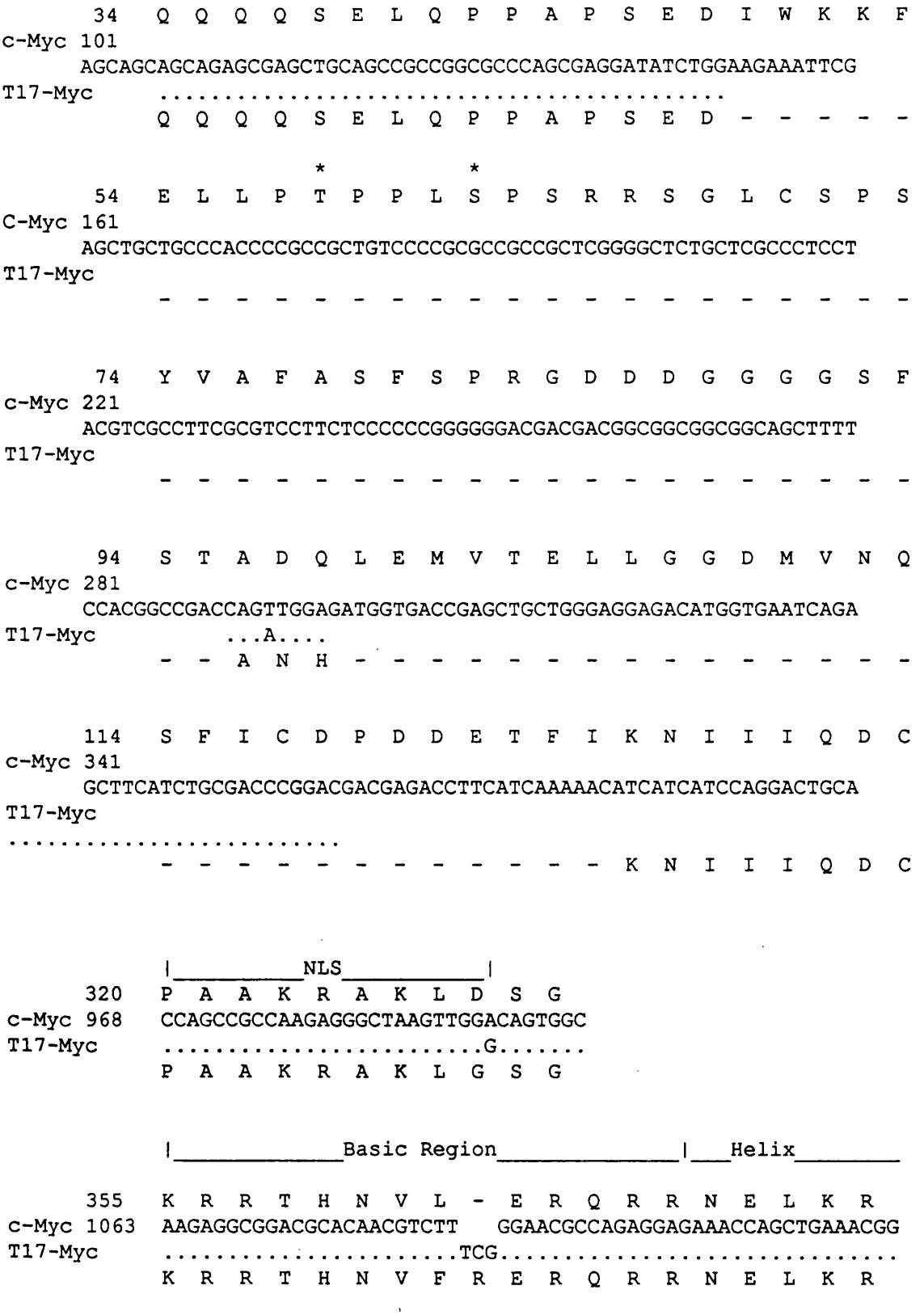


Figure 3.6 DNA sequence and protein translation of T17-Myc mutations.

Phosphorylation sites are marked *. NLS, nuclear localization signal.

CHAPTER 4

Chapter 4 Dissociation of the transforming and apoptosis functions of T17 *myc* in a primary chick cell assay

Chapter 4: Dissociation of the transforming and apoptosis functions of T17 *myc* in a primary chick cell assay

4.1 Introduction

Transformation assays are frequently used to ascribe oncogenic potential to a particular gene product, or to a particular type of mutant gene product (203). However, one limitation of most systems involved is that they often require cells already primed for susceptibility to transformation. Assays which have been used to assess the transforming potential of *myc* often employ this initial "hit" technique, with the *ras* gene being a frequent target for the first genetic lesion (209). Land *et al* used the *ras* gene as a cooperating gene in transformation to demonstrate that c-*myc* could transform cells as efficiently as v-*myc* (34). However, this experiment utilised a c-*myc* derived from a mouse plasmacytoma which was modified to remove the immunoglobulin gene (34), therefore this *myc* may well have been mutated. The first demonstration that a normal c-*myc* was able to cause transformation was given by Martin *et al* (210) using quail embryo fibroblasts. To gauge the potential of the T17 mutant to cause transformation in an *in vitro* system, the chick embryo fibroblast (CEF) assay was employed. This assay was chosen as primary avian cells are sensitive to transformation by deregulated Myc, without the requirement of a cooperating gene such as *ras* (210).

To enable the expression of the feline *myc* genes to take place in avian cells, a retroviral vector containing the LTR regions of the avian erythroblastosis virus was used (174). This vector also contains the neomycin resistance gene, allowing transfected cells to be selected using the drug G418 (neomycin). Figure 4.1 is a graphical representation of how the vectors were constructed. Following transfection with the vectors and subsequent drug resistance selection, cells were assayed for growth rate and their ability to grow in an anchorage independent manner, the latter probably being the best measure of transformation.

One of the most intriguing facets of the *myc* gene is its apparent dichotomous ability to promote either cellular immortalisation or cell death. Much of the early work on *myc* focused on the ability to promote cell survival and enhance transformation, but in recent years there have been numerous studies investigating the ability of *myc* to promote programmed cell death or apoptosis. This latter function has been shown to occur when fibroblasts and myeloid cells which overexpress Myc are depleted of growth and or survival factors (63,88). Evidence also exists of a role for Myc in the mechanism of apoptosis in T-cells, via the T-cell receptor (TCR), where immature thymocytes and hybridomas can be induced to undergo apoptosis when stimulated through the TCR. However, the apoptosis pathway can be prevented by *myc* antisense oligonucleotides, whereas oligonucleotides with the same base content but in random order had no effect. Prevention of apoptosis was achieved without interfering with other cellular functions, including cytokine production (89). Further work demonstrated the need for functional Myc/Max heterodimers in the induction of apoptosis by Myc (97,98).

A detailed methodology of the techniques used in the chick embryo fibroblast assays is given in section 2.2.8.

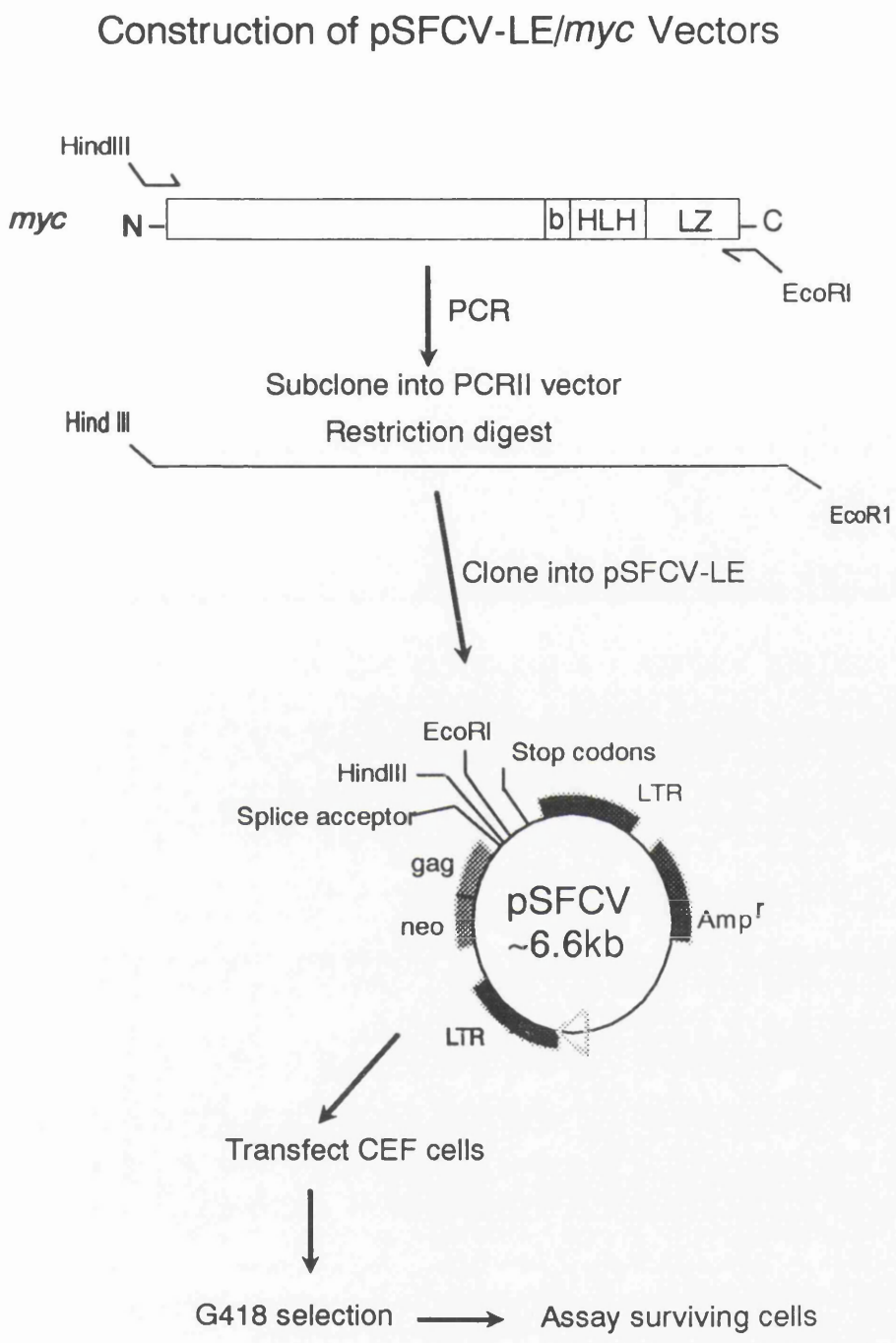


Figure 4.1

Diagrammatic representation of how the *myc*-containing retroviral vectors were constructed and used.

4.2 Results

4.2.1 Growth curves of transfected CEF cells

Post G418 selection cells were assayed as described (Section 2.2.8.4), from the graph (Figure 4.2) it is immediately clear that the T17 Myc cells grow at the same rate as the control cells. However, c-Myc transfected cells have a substantially increased growth rate in comparison to control cells. At 24 hours after the beginning of the experiment no cell type had more cells per dish than the original number plated, this was most likely due to cells dying after trypsinisation of the starting culture before plating. The differential growth rate manifested itself after 48 hours, by which time the c-Myc expressing cells were noticeably more dense than either control cells or T17-Myc expressing cells.

4.2.2 Transfected CEF cells produce the exogenous Myc protein

Western immunoblot analysis confirmed that the CEF cells express the Myc protein from the retroviral constructs as shown in figure 4.3. Therefore, effects on cell growth, transformation and apoptosis can be attributed to the exogenous Myc protein. High levels of T17 Myc protein, previously shown to be a feature of the T17 tumour cell line, were again noticeable in the chick cells as a stable product at approximately 50kD.

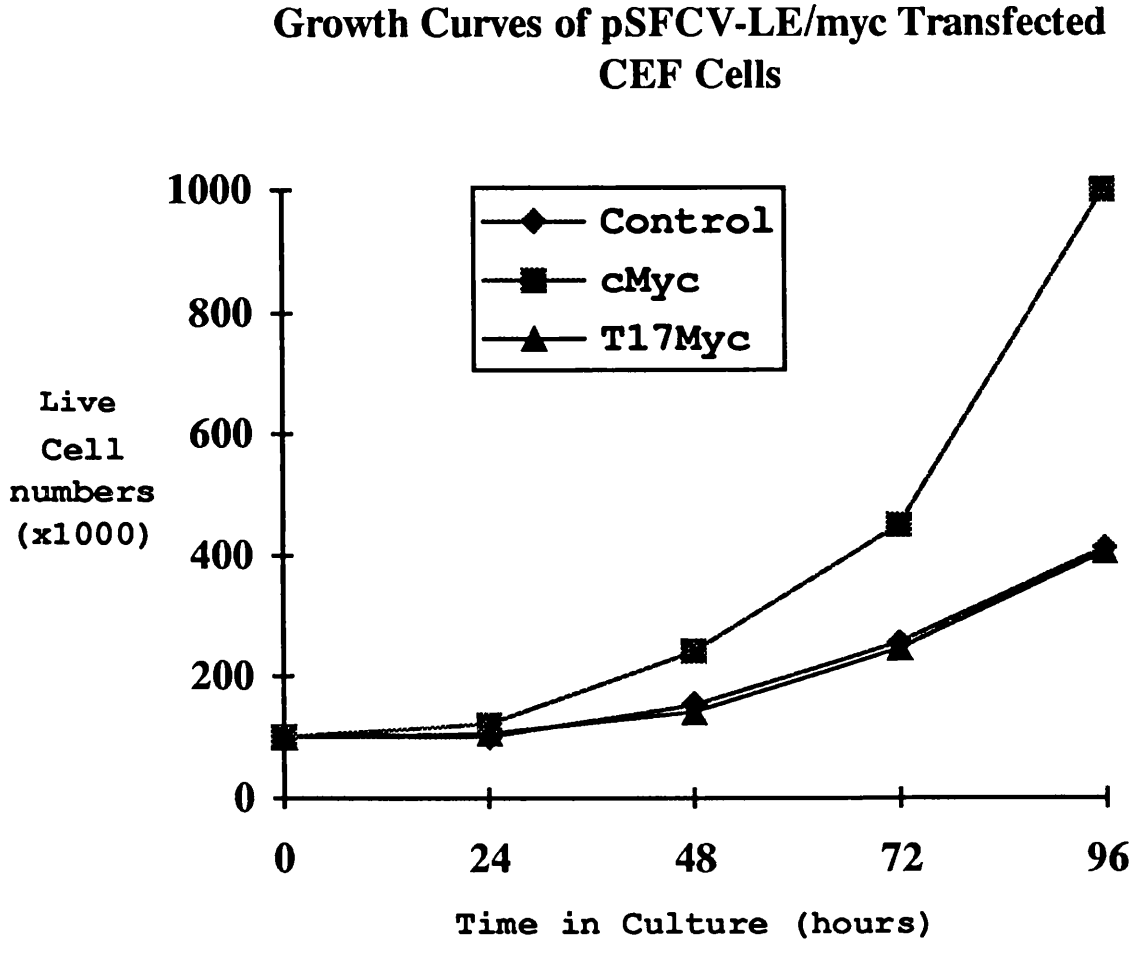


Figure 4.2

Control cells in this assay were CEF cells which had been transfected with vector alone and selected with G418. By the end of the culture period c-Myc producing cells were generally confluent in the 35mm dishes, while in all experiments the control and T17 Myc cells had substantial space for further growth.

4.2.3 Overexpression of feline cMyc, but not T17 Myc, transforms CEF cells

Markers of cellular transformation induced by Myc in CEFs include an increased growth rate and altered cellular morphology (211). However, anchorage independent growth of adherent cells is a more readily quantifiable measure of transformation. Hence growth in soft agar is recognised as an indicator of a cell having achieved a transformed phenotype (34). Results of this assay are shown in figure 4.4, which confirms that the rapidly growing, cMyc producing cells, are also the only cells to be transformed in this assay.

4.2.4 Feline cMyc, but not T17 Myc, induces apoptosis under culture conditions of low serum

Deprivation of serum caused cMyc producing cells to undergo apoptosis, whereas the T17 Myc expressing cells were not susceptible to an increase in this type of cell death. From figure 4.5 it can be seen that the T17 Myc cells did not display any increase in apoptosis above the control cells. Control gating on this figure was carried out on the serum positive cells of each cell population. The control for background fluorescence was to put cells through the same biotin-dUTP labelling protocol as the cells shown, however, control cells were not incubated with terminal transferase. The R1 gate denotes the cell population which do not display fluorescence above background. Gate R2 shows cells which have incorporated biotin-dUTP into DNA strand breaks, the percentage of cells in R2 of each cell population is given on the figure.

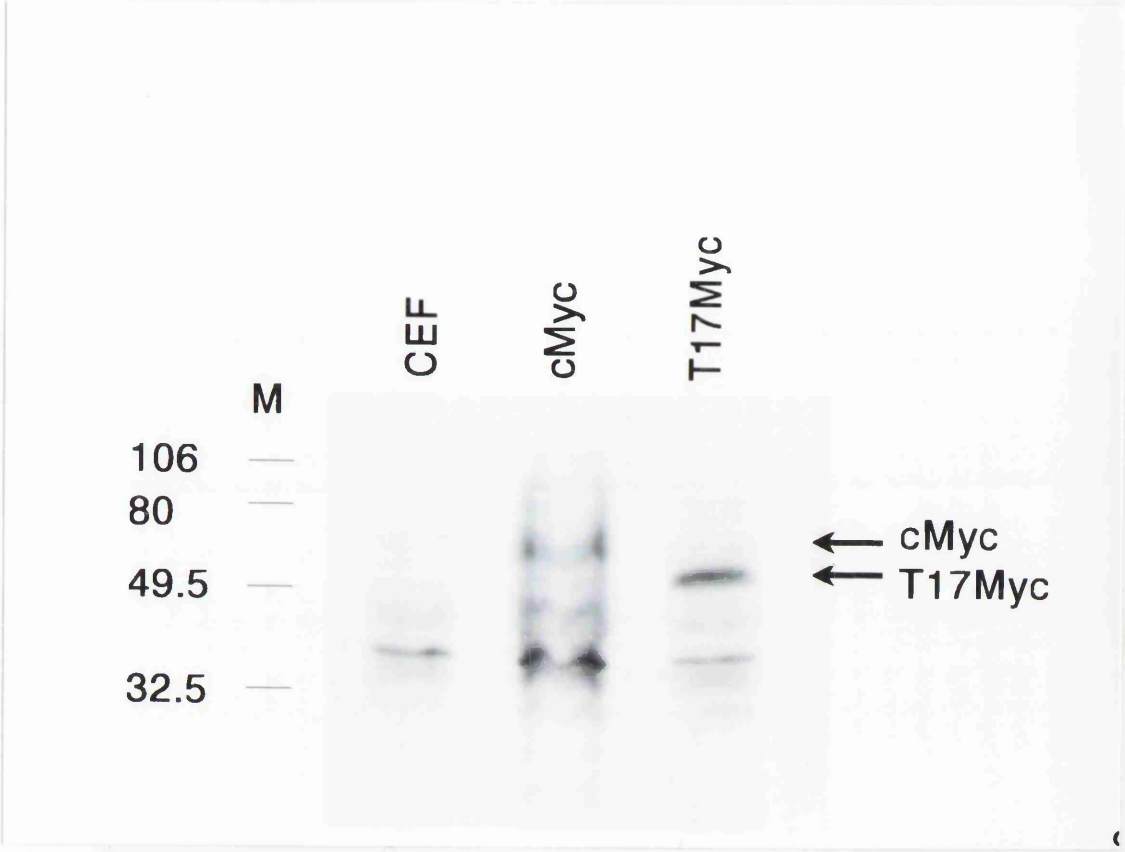


Figure 4.3 Western immunoblot of transfected CEF cells.

CEF, cells transfected with vector alone.

This Figure demonstrates that excess Myc is present in the appropriate cells, and that the T17 transfected cells contain a higher mobility protein of ~50kD.

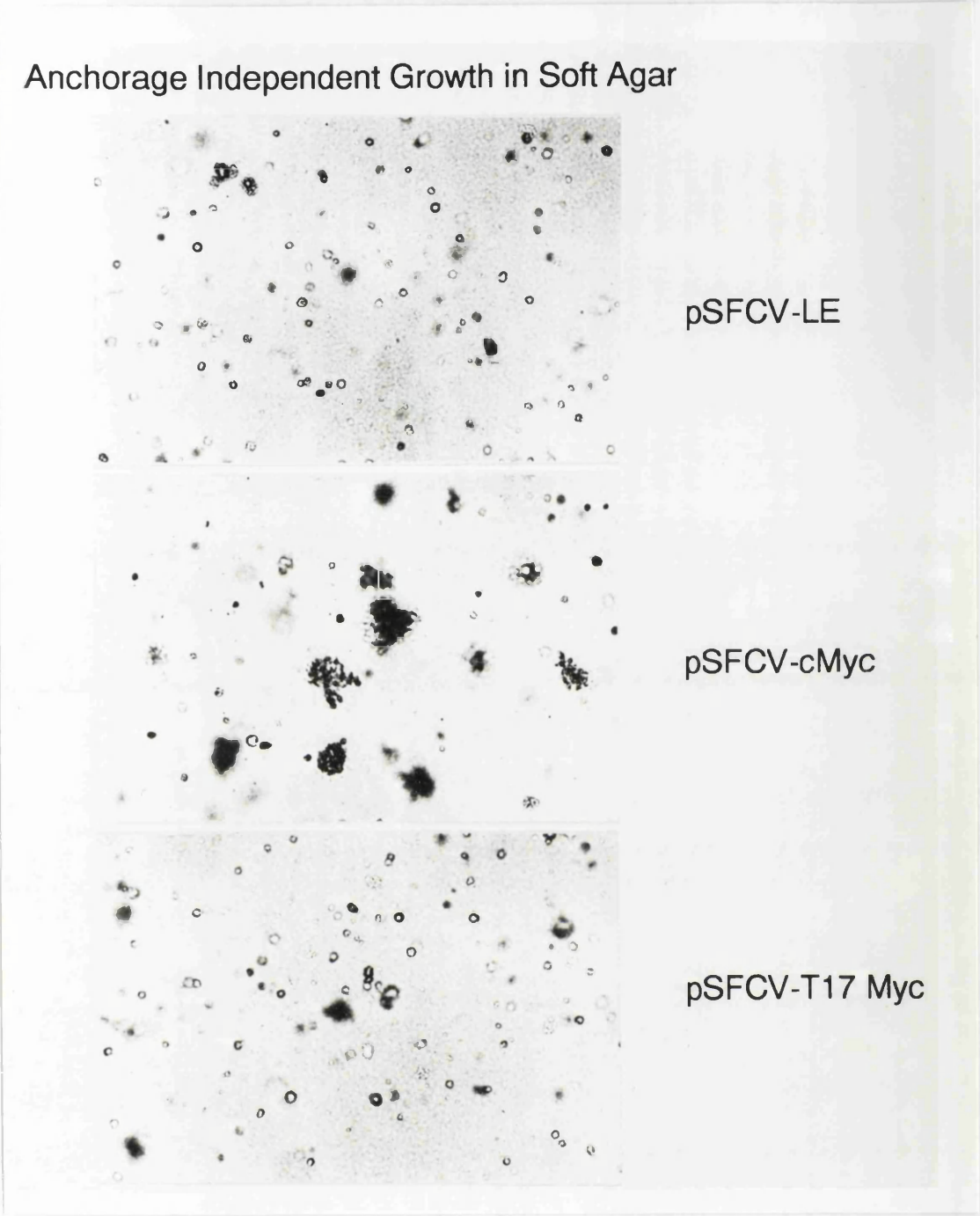


Figure 4.4 Growth in soft agar transformation assay of transfected CEF cells (Magnification X100).

This Figure demonstrates that only the c-Myc expressing cells are capable of growth in soft agar.

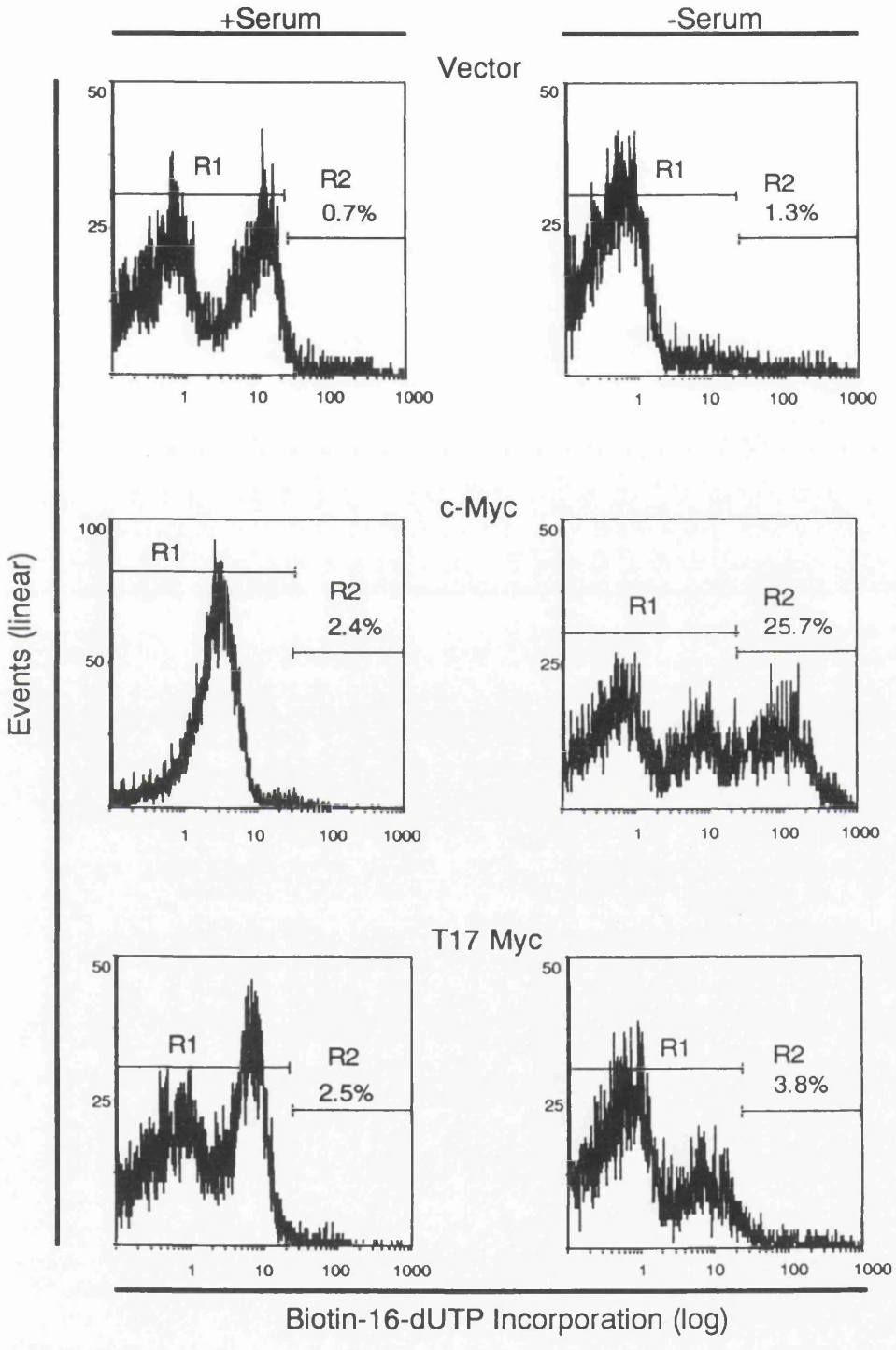


Figure 4.5

Apoptosis analysis by terminal transferase labelling of DNA strand breaks. R1 denotes cells not undergoing apoptosis, R2 is the percentage of cells incorporating biotin-dUTP.

4.3 Discussion

Overexpression of Myc usually correlates with a loss of growth control, leading to transformation of the affected cells. Indeed this occurs with feline c-Myc in the CEF assay, while the T17 Myc protein does not have any discernible effect, even though there is a large amount of the mutant protein present in the cells. These data suggest that T17 Myc might not be functional in chick embryo fibroblasts, i.e. although present, the protein cannot interact with other parts of the transcriptional machinery. However, this does not seem likely, as the mutant maintains the ability to interact with Max and bind to the consensus E-box motif (chapter 6). It seems more likely that the T17 mutant permits the normal growth of CEF to take place despite high concentrations of the protein which can complex with Max. This finding suggests that the regions deleted in the N-terminus are required for the increase in growth rate and apoptosis functions, but are dispensable for the normal cycling of these CEF cells. Otherwise we would expect a slower increase in cell number than the control cells. Thus the presence of the deletion mutant is not deleterious to the normal cycling of these cells. Indeed a transformation defective deletion mutant lacking amino acids 91-137 of MC29 v-Myc has been shown to prevent transformation when cells are superinfected with wild type MC29 virus (212).

The first demonstration that mutant *myc* genes could affect different cell types in different ways, came from work on the MC29 v-*myc*. Early observations of the MC29 transformed quail fibroblast cell line, Q10, noted that smaller *gag* gene-related products were synthesized after prolonged periods in culture (213). Three spontaneously occurring mutants were isolated, 10A, 10C and 10H, which synthesized fusion products of MWs 100,000, 95,000 and 90,000 respectively. These mutants were found to be competent to transform CEF cells, but had a much reduced ability to transform macrophages (213). Restriction enzyme mapping of 10A, 10C and 10H, showed that they contained overlapping deletions of 200, 400, and 600 base pairs respectively, which mapped to a region around a Cla I site near the middle of the *myc* sequence (214).

Later work focused on defining regions of v-Myc which are important for the transforming potential in different cell types. Using deletion mutants created by restriction digest of plasmids containing the MC29 genome, several clones were identified with changed transformation specificities (202). Deletion of most of the *gag* sequence was shown to have no effect on the ability of a mutant to cause transformation of either fibroblasts or bone marrow cells, but mutation of the *myc* sequence resulted in changed growth parameters. Mutants with deletions between amino acids 200-300, had lost the ability to transform bone marrow cells, but retained the ability to cause transformation of fibroblasts (202) (Table 4.1).

Other work which created deletions at the N-terminal of the *myc* coding sequence found a different set of results. In this work it was again found that deletion of the *gag* sequences did not affect transformation capacity (212). However, deletion of the *myc* sequences covering those lost in the N-terminal domain of T17-Myc allowed transformation of macrophages, but greatly reduced the ability of the mutants to transform fibroblasts (212) (Table 4.1). Also notable is that a mutant with deletion of amino acids 1-42, which are retained in T17-Myc, was initially presumed to be transformation defective in culture, until cells were plated in soft agar, where large macrophage colonies were formed (212).

These data indicate that cell type specificity is likely to play a substantial role in the ability of any mutant Myc to transform. The inability of the T17-Myc mutant to transform CEF cells is consistent with the data from the MC29 deletion mutants covering the equivalent domain of Myc.

However, factors other than cell type specificity may be involved, as the same cell types from different species have been shown to differ in sensitivity to transformation by mutant v-*myc* genes. Again, deletion mutants were used to demonstrate the effect. Deletion of as few as 11 amino acids from middle of v-Myc prevented transformation of

chicken bone marrow cells and peripheral blood macrophages, whereas deletion of up to 200 amino acids covering the same region still allowed efficient transformation of quail macrophages (215). The same mutants could induce short-latency tumours in newborn and one week old Japanese quail, with a similar efficiency to wild type MC29. Further, restriction digests and Southern analysis of tumour material showed that recombination between v- and c-*myc* had not occurred, as restriction patterns of tumour DNA showed smaller fragments in the mutant induced tumours compared to wild type MC29 induced tumours (215). Thus, both cell type specific factors and/or species specific factors may be important when trying to measure the transforming capability of c-Myc.

These findings prompted the question, "can chimaeric *myc* retrovirus constructs dissociate differences in function between the N-, and C-terminal mutations?" This question is addressed in chapter 5.

Table 4.1 Ability of avian v-*myc* mutants to cause growth in soft agar. It should be noted that the studies from which this table has been compiled (from (202,212)) included other mutants, including insertion mutants, which are not referred to, but which support the conclusions drawn.

Deletion (amino acids)	**Fibroblast growth in soft agar**	**Macrophage growth in soft agar**
1-42	-	+++
43-57	-	++
58-84	-	++
85-90	-/+	+
91-137	-	-
138-245	-	++
220-239	+	-
239-249	+	-
220-279	+	-
205-288	+	-

Table 4.1

CHAPTER 5

Chapter 5: A chimaeric Myc which allows transformation and apoptosis does not increase growth rate

Chapter 5: A chimaeric Myc which allows transformation and apoptosis does not increase growth rate

5.1 Introduction

The lack of a discernible phenotype for T17 Myc in the CEF assay prompted the question as to whether any phenotype might be observable, if the N-terminal and C-terminal mutations were separated and used in the CEF assay. Therefore, to further characterise what role the T17 Myc mutations might play in cellular growth and transformation, chimaeric *myc* constructs were used in the same CEF assays outlined in the previous chapter. The chimaeras consist of 296 amino acids from the c-Myc N-terminus fused to the v-Myc C-terminal 144 amino acids, and the equivalent 222 amino acids from the v-Myc N-terminus fused to c-Myc C-terminal 143 amino acids. These constructs are called c/v-Myc and v/c-Myc respectively. The methods used to construct the chimaeric genes used in this set of experiments are described in chapter 2.2.8.

5.2 Results

5.2.1 Both N- and C-terminal domains of T17-Myc are deficient in growth rate enhancement of CEF cells

Analysis of the growth curves shown in figure 5.1 demonstrates that neither chimaeric Myc construct had any effect on the growth rate of transfected cells, in comparison to control cells transfected with the vector alone. Several repeats of the growth rate experiment failed to show an increased growth rate of CEF cells, with both chimaeras having the same growth curve as the vector control cells. Thus, the C-terminal basic region mutation confers a change in the growth rate phenotype in comparison to the wild type c-Myc as shown in figure 4.2.

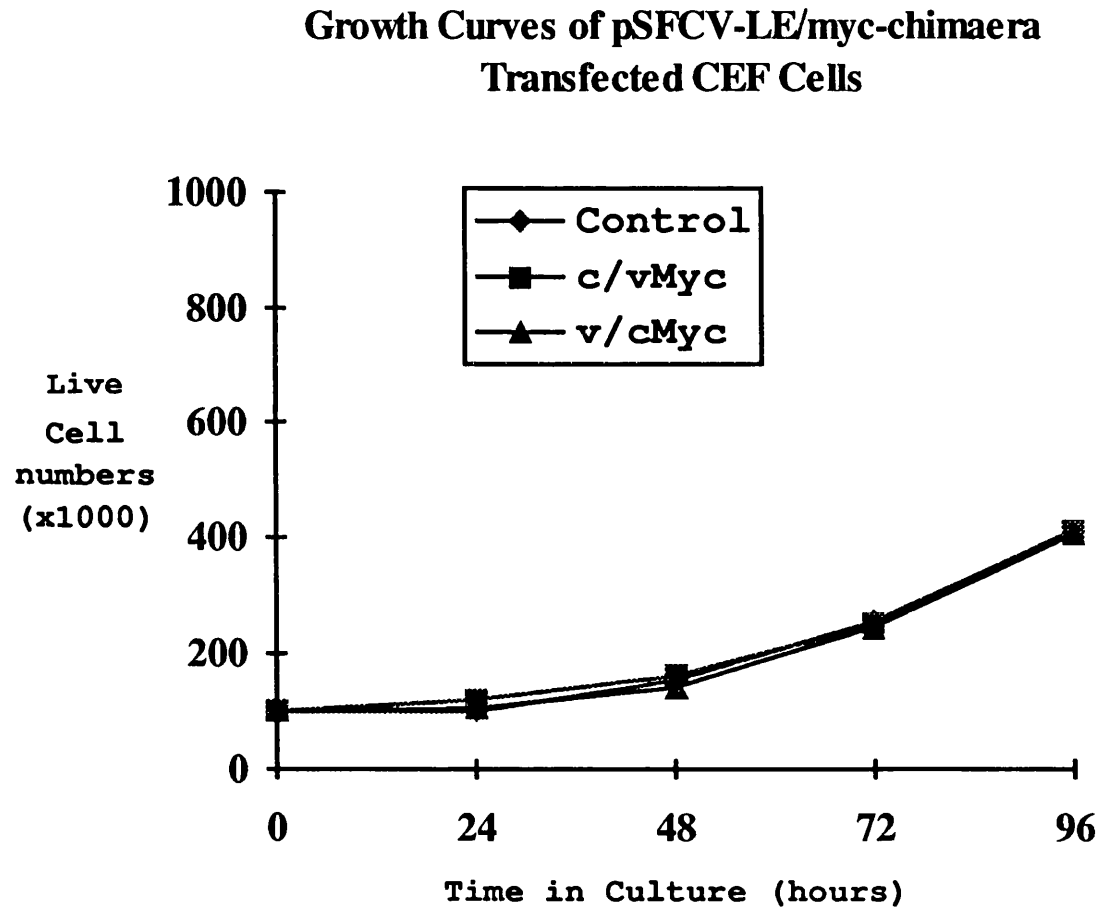

Growth Curves of pSFCV-LE/myc-chimaera
Transfected CEF Cells
Control
c/vMyc
v/cMyc
Live
Cell
numbers
(x1000)
1000
800
600
400
200
0
0
24
48
72
96
Time in Culture (hours)

Figure 5.1

5.2.2 Western blot analysis confirms that exogenous Myc is present

Figure 5.2 demonstrates that the exogenous Myc protein is in fact present in the cells transfected with the viral constructs carrying the chimaeric *myc* genes. This figure further shows that Myc proteins present are of the expected molecular weights for the chimaeric translation products, i.e. ~62 kD for c/v-Myc and ~50 kD for v/c-Myc. Therefore, an inability to increase the growth rate of CEF cells is not due to a failure of the retroviral constructs to drive expression.

5.2.3 A c/v-*myc* chimaera transforms CEF cells

The ability to grow in soft agar has previously been described (4.3.3) as a means of demonstrating the transformed phenotype. Use of the chimaeric constructs demonstrates that the N-terminal region of the feline Myc protein is required for transformation as measured by anchorage independent growth in soft agar (Figure 5.3). The number of colonies which grew out in this assay was consistently lower for c/v-Myc than for intact c-Myc in the same assay, giving approximately 10% of the number of colonies of c-Myc transfected cells. This must be due to the basic region mutation present in the chimaeric protein, which also accounts for the lower growth rate.

5.2.4 Analysis of apoptosis in c/v-, and v/c-Myc chimaera expressing CEF cells deprived of serum

As demonstrated in Figure 5.4 only the c/v-Myc chimaera induced apoptosis when CEF cells were deprived of serum. Although there was a smaller number of cells undergoing apoptosis with the c/v-Myc chimaera compared to the native c-Myc (figure 4.5), this result is consistent with the slower growth rate of the chimaera expressing cells.

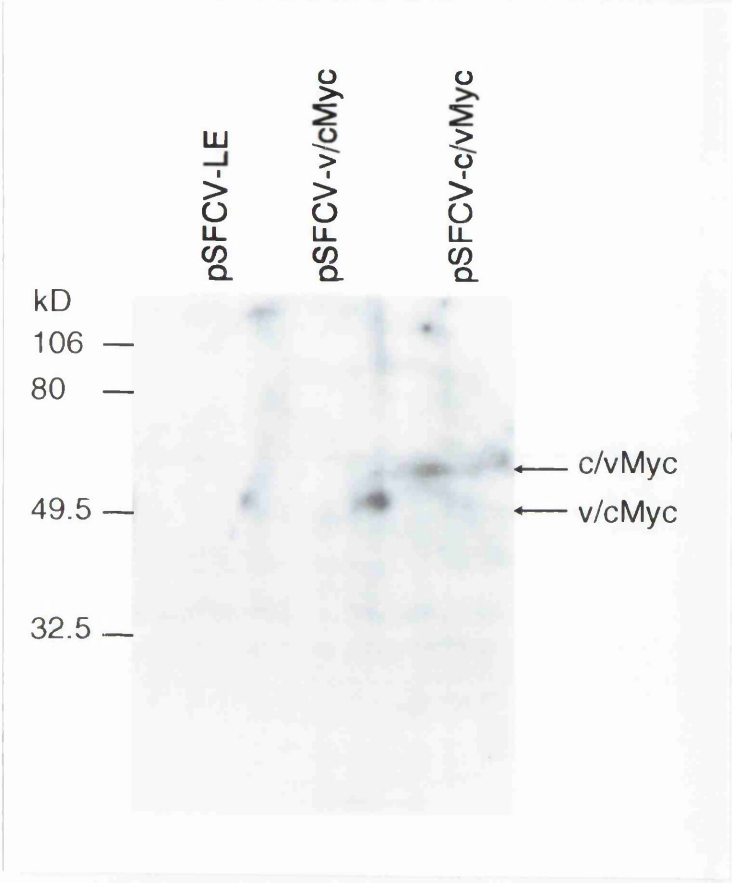


Figure 5.2 Western immunoblot of transfected CEF cells.

This figure demonstrates that the chimaeric proteins are expressed.

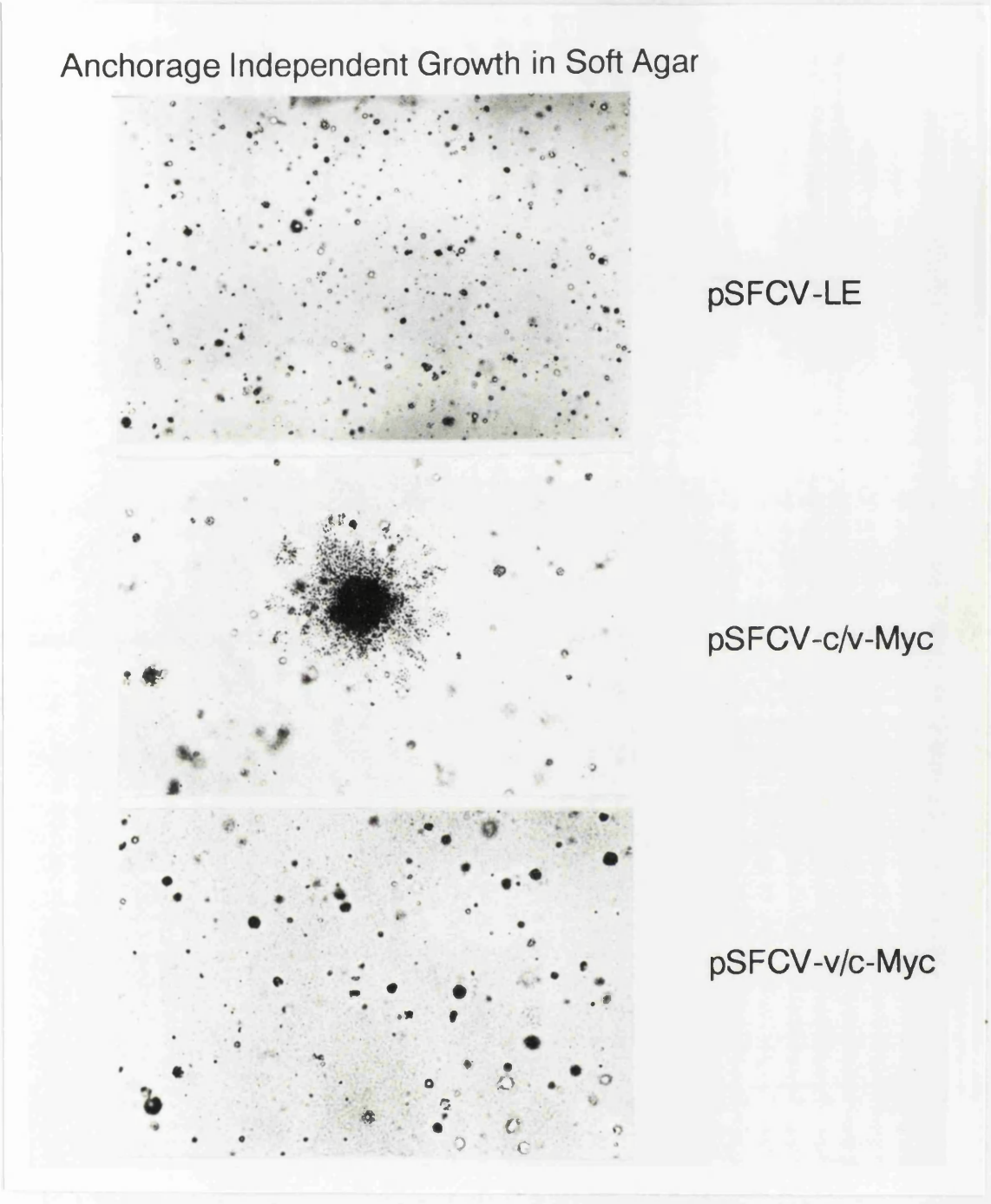


Figure 5.3 Growth in soft agar transformation assay of CEF cells transfected with the Myc chimaeras. Magnification X100 for chimaeras, X40 for control cells.
Only the c/v-Myc chimaera gave growth in soft agar. Although the colony displayed is larger than c-Myc colonies in Figure 4.4, this is likely to be due to a lack of growth factors for the c-Myc colonies compared to chimaeric colonies, which gave far fewer colonies per experiment.

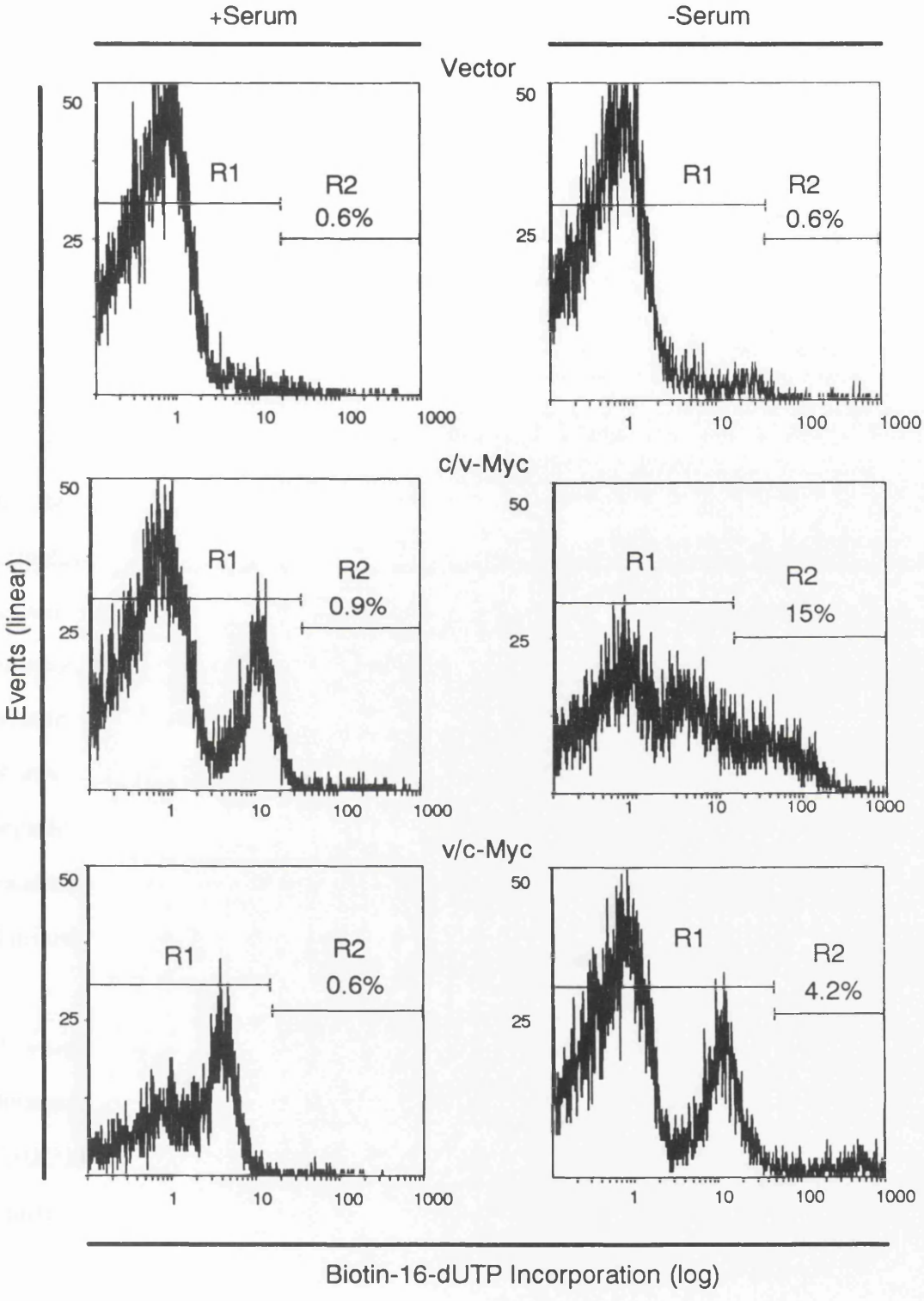


Figure 5.4 Apoptosis analysis by terminal transferase labelling of strand breaks. R1 denotes cells not undergoing apoptosis, while R2 gives the percentage of cells incorporating biotin-dUTP above background, i.e. undergoing apoptosis.

5.3 Discussion

The ability of a chimaeric Myc, containing a basic region mutation, to induce apoptosis on serum deprivation, and to transform cells while failing to increase growth rate is a novel finding. Although other groups have demonstrated the requirement for an intact N-terminal domain in transformation and apoptosis assays (63,203), no basic region mutants have come to light which display this failure to increase growth rate. Several experiments carried out by myself and Dr. D. Crouch confirmed that this finding is repeatable. This result does not contradict the findings of the last chapter but does show that the failure to increase growth rate in CEF cells may be due to both the N-, and C-terminal mutations, especially as the v/c-Myc chimaera also failed to increase growth rates of transfected cells. What is suggested by the work in this chapter is that differing functions which Myc can play a role in, transformation, apoptosis, and growth rate, may be activated by different gene products under the control of Myc. It is unlikely that the observed effects are caused by the Asp-329 to Gly substitution which borders the peptide sequence PAAKRAKLD this being equivalent to the human c-Myc nuclear location signal (PAAKRVKLD) (282), as deletion of this region of v-Myc did not diminish its transforming activity (283).

Previous mutational analysis of the C-terminal domain which disrupted the BR-HLH-LZ domains of Myc had proven to be incompatible with a transforming phenotype (202,211,212). However, although the ability to dissociate growth rate from transformation is novel, other C-terminal mutations which can affect Myc function have been described. One such avian Myc mutant carries a deletion of the C-terminal 7 amino acids (c-MycΔ7) (211), while another avian mutant - termed S90.9 - has a 9 amino acid deletion at the C-terminal (216). Both mutations result in the loss of one of the heptad repeat leucine residues. The transforming phenotype of these mutants has been shown to be intermediate between the wild type c-Myc, and leucine zipper deletion mutants which displayed no change in phenotype from control cells (211,216). Although the

mutants displayed increased growth rate and loss of density inhibition, there was an impaired ability to grow in soft agar. Although the S90.9 mutant appeared to be only partially effective at transforming avian fibroblasts, this mutant showed no defect in its ability to transform avian macrophages (216), thus demonstrating that the ability of a mutant v-Myc to transform a particular cell type is not necessarily a good indicator as to its ability to transform other cell types.

Later work using the c-MycΔ7 mutant further characterised the phenotype as being unable to suppress expression of *myo*D and related muscle differentiation specific genes, although c-Myc could suppress the same genes when overexpressed (217). Although the effects attributed to c-MycΔ7 have been purported to show that Myc can affect gene transcription via a Max-independent pathway, it has not been excluded that the effects described are due to differing levels of effective Myc/Max complexes, especially as the c-MycΔ7 mutant was only half as active as c-Myc in a Max-dependent transcription assay in yeast (176).

The results from this chapter and the previous chapter are summarised in table 5.1. These results indicate that the BR mutation (p.60) is worthy of further study in isolation. Therefore in an endeavour to attribute special characteristics to the BR mutation, studies described in chapter 6 were undertaken to investigate the biochemistry of the T17-Myc BR mutation.

Table 5.1 Properties of chimaeric feline *myc* gene constructs in CEF cells.

Myc construct	Growth rate increase	Growth in soft agar	Induction of apoptosis
c-Myc	+	+	+
T17-Myc	-	-	-
c/v-Myc	-	+/-	+
v/c-Myc	-	-	-

CHAPTER 6

Chapter 6 Heterodimerisation With Max and Transcription in Yeast are not Detectably Affected by the T17 Basic Region Mutation

Chapter 6 Heterodimerisation With Max and Transcription in Yeast are not Detectably Affected by the T17 Basic Region Mutation

6.1 Introduction

In order to investigate the ability of the basic region mutation to interfere with Myc/Max heterodimerisation and DNA binding, the C-terminal 115 amino acids of c-Myc and 116 amino acids of T17-Myc were expressed as glutathione-S-transferase (GST) fusion proteins (175). Expression of proteins of interest as GST fusion proteins allows rapid purification of the desired protein by binding to S-linked glutathione agarose beads and subsequent washing to get rid of contaminants, before elution from the beads with reduced glutathione. These fusion proteins were then used with GST-Max fusion proteins in electrophoretic mobility shift assays (EMSA). The EMSA or 'gel shift' is an *in vitro* method of determining whether proteins can bind to radioactively labelled oligonucleotide DNA (182). To exclude species differences in the Max protein as a source of anomalous results, cDNA clones of the feline *max* and *max*9 transcripts were isolated and sequenced (218). Subsequently feline *max* was expressed as a GST fusion construct for use in the EMSA.

A yeast assay was also employed, to gauge what effect the BR mutation would have on transcription in a reporter system which required Myc/Max heterodimerisation (141). In this assay the BR-HLH-LZ of Myc is linked to the yeast PHO4 transactivation domain. The yeast transcription factor PHO4 contains a BR-HLH motif which recognises the same CACGTG consensus binding motif as Myc (120). In the case of PHO4 the target gene is known to be the acid phosphatase PHO5, which is positively regulated by PHO4 (219). Indeed the basic region of c-Myc and PHO4 are interchangeable with no apparent loss in specificity (120). However, this assay completely removes the PHO4 BR-HLH and replaces it with the Myc BR-HLH-LZ which activates the reporter construct only in conjunction with Max (176). Therefore using a reporter construct,

which contains the PHO4 target upstream activating site (UAS) from the *in vivo* target PHO5 linked to LacZ, allows quantitative as well as qualitative data to be collected (176), thus allowing a direct comparison to be made between the c-Myc and T17-Myc BR-HLH-LZ domains.

6.2 Materials and Methods

6.2.1 Cloning and sequencing of the feline *max* and *max*9 transcripts

The high degree of homology amongst previously published *max* sequences (115,220) allowed primers to be designed for sequences which are identical in human and mouse, primer sequences are :-

5'-GGA AAT GAG CGA TAA CGA TG-3'

5'-GGC TTA GCT GGC CTC CAT CC-3'

5' and 3' ends respectively.

3.5μg of total RNA isolated from normal feline thymus using the RNazol method (Biogenesis), was subjected to first strand cDNA synthesis using a commercial kit (Pharmacia) and including 175pmol of the 3' primer. 160pmol of the 5' primer was added to the reaction for the subsequent polymerase chain reaction step. The conditions for the 30 cycles of PCR reaction were denaturation at 94^{o}C for one minute, annealing at 55^{o}C for one minute and elongation at 72^{o}C for one minute. Two reaction products of approximately 490bp and 460bp were observed, when the reactions were subjected to gel electrophoresis on a 6% polyacrylamide gel (Scotlab). These fragments were cloned into the PCR cloning vector pCR II using the TA kit (Stratagene). Each clone was identified by restriction enzyme digest using EcoRI, followed by analysis on a 6% polyacrylamide gel. Clones carrying inserts of each size were sequenced on both strands using a long read Sequitherm cycle sequencing kit (Epicentre Technologies), with an infrared labelled M13 forward or M13 reverse primer (Li-Cor) and sequenced on a Li-Cor model 4000 automated sequencer.

Feline *max* was expressed as a glutathione-S-transferase (GST) fusion construct (Section 2.2.10.1) and used along with GST-BR-HLH-LZ fusion proteins (Section 2.2.10.2) in electrophoretic mobility shift assays (EMSA) as detailed in Section 2.2.3.

6.2.2 Construction of PHO4-Myc chimaeras and β-galactosidase assays

The PCR was used to generate C-terminal BR-HLH-LZ fragments of c-*myc* and T17-*myc*, containing the restriction sites XhoI and ClaI at the 5' and 3' ends respectively. These restriction sites allow in-frame directional cloning of the digested products into the vector pTZ which contains the transactivation domain sequence for PHO4. Following ligation into pTZ the PHO4-*myc* construct is excised using Bam HI and this sequence is subcloned into a unique Bgl II site in the yeast expression plasmid pMA132-a. The orientation of the construct was checked by double digestion with Xho I and Bam HI, as the subcloning of the Bam HI fragment from pTZ into the Bgl II site of pMA132-a destroys both the Bam HI and the Bgl II sites, the only Bam HI site which cuts, is a site in pMA132-a which is 3' to the insert. Thus constructs in the correct orientation were smaller, i.e.~1200bp as opposed to ~1500bp. The pMA132-a plasmid contains the promoter sequence of the yeast phosphoglycerol kinase gene which gives high levels of transcription (176). Also encoded by this plasmid is the tryptophan resistance gene.

The primer sequences used to generate the *myc* portion of the constructs were:-

5' Primer 5'-CAG ACC TCG AGG AGA ACG ACA AG-3'

3' Primer 5'-CTG GAT CGA TCC TCC CTC TAA TAG GTG G-3'

The Xho I and Cla I sites are underlined on the 5' and 3' primers respectively. These primers generated 273 bp (276 for T17) of *myc* coding sequence, including the stop codon. The 3' primer overlapped the end of the coding sequence allowing the *myc* stop codon to terminate translation of the chimaeric protein.

Human *max* and *max*9 cDNAs had previously been cloned into the Bgl II site of the μ plasmid pKV701 downstream of the inducible *GAL* 10 promoter by Dr. Goding's group (176), and sub-cloned, together with the *GAL* 10 promoter, into the plasmid pRS314, for use in this assay.

The yeast strain used in the assay was Y704 which lacks endogenous PHO4 activity (120), and was transformed and assayed for β-galactosidase activity by Dr. Goding using the vectors I had constructed. Briefly, transformed yeast were selected on yeast glucose minimal agar plates supplemented with appropriate amino acids. Colonies were picked into 6 ml of fresh glucose minimal medium and grown until they reached stationary phase, then cultures were centrifuged and pellets resuspended in 1 ml of galactose minimal medium and 100 μl used to inoculate 6 ml of fresh galactose minimal medium. Following 24 hours induction, cells were harvested, washed once in water, resuspended in 100 μl of 0.1M Tris pH 7.5, 0.05% Triton X-100, and frozen on dry ice. Subsequently 30μl aliqouts were assayed for β-galactosidase activity. Units were calculated using the formula A_{420}/A_{600} X 1000/CVt where A_{420} is the absorbance at 420nm, A_{600} is the density is the density of the cell suspension of 6ml of galactose medium, V is final volume of cells used in ml (0.030) and t is time of reaction in minutes (176). Figure 6.1 is an illustration of the constructs used in this assay.

C-terminal Myc Transactivation assay in Yeast

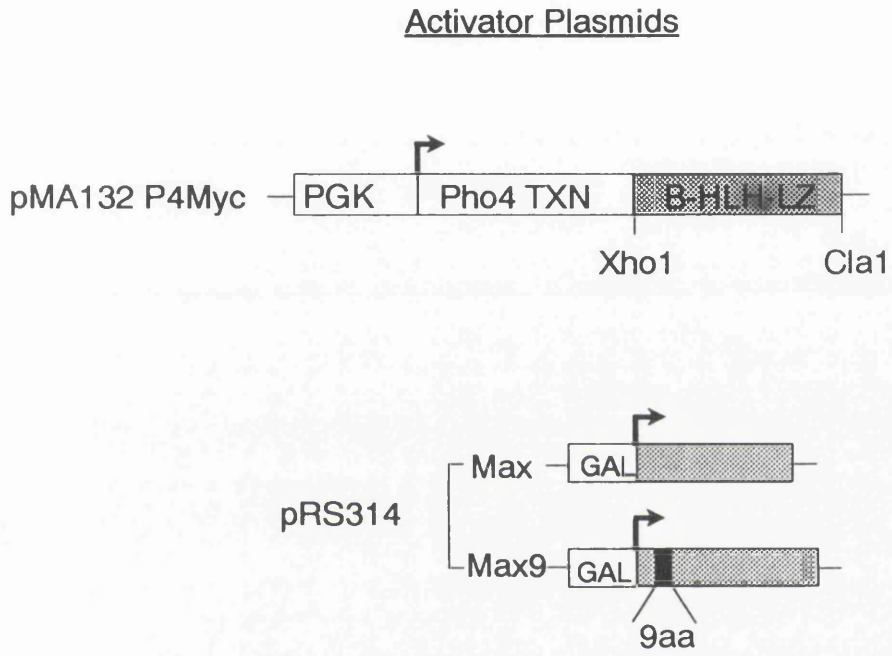


Figure 6.1

6.3 Results

6.3.1 Feline Max is identical to human Max at the amino acid level

Figure 6.2 shows the nucleotide sequence of feline *max* with the predicted single letter amino acid code above the triplet codons. From this figure it can be seen that the larger transcript encodes a peptide of 160 amino acids, while the shorter transcript lacks 9 of these codons. The feline *max* DNA sequence shows 98% identity to that of human and 95% to mouse. The feline and human proteins are identical while Myn is 98% related at the amino acid level (115,220).

This sequence data appears in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession number, D37786.

6.3.2 Analysis of recombinant GST-fusion proteins

Coomassie blue staining of a SDS-PAGE gel with GST-agarose purified proteins demonstrates that the purified GST-Max protein construct is very stable (Figure 6.3). In contrast, the same gel demonstrates that GST-BR-HLH-LZ recombinant proteins are unstable, with approximately 90% of the purified protein being degraded (Figure 6.3). However, western immunoblot analysis (Section 2.2.10.4) of the GST-BR-HLH-LZ protein demonstrates that the expressed proteins are correctly translated (Figure 6.4). Several methods were tried in an effort to produce a more stable GST-Myc construct, including IPTG induction for less time, induction at a lower temperature than 37^{o}C, and various protease inhibitors, however, no better preparations were obtained.

6.3.3 The GST-T17 Myc C-terminal construct, in cooperation with GST-Max, efficiently alters the mobility of oligonucleotides containing the consensus CACGTG motif in an EMSA

The CM1 oligo was based on that used by Blackwell *et al* to show that the consensus binding site for Myc is CACGTG (114). Gel retardation of the CM1 oligo by the GST-T17 Myc proved to be every bit as efficient as that by GST-cMyc, as demonstrated in Figure 6.5. Two specific retardation bands are present due to BR-HLH-LZ portions of protein, which are not attached to GST being present in the binding reaction. However, other lines of evidence have led to the conclusion that bases flanking the core six bases can affect the binding of Myc/Max to DNA (221-223). Therefore, it was felt that the use of a probe which had previously been shown to discriminate between proteins which recognise the same core CACGTG motif, due to bases outwith the core, should also be used in the EMSA. Hence the P2 probe (223) was chosen in an attempt to detect potential differences in binding between the mutant and the wild type basic regions. From Figure 6.6 it is clear that there is no gross difference in the binding of the two Myc constructs to the P2 Probe. To look at possible differences in the avidity of the two Myc proteins for the CM1 oligo, radioactively labelled CM1 was competed out using unlabelled 'cold' P2 oligo. Again no significant difference was evident, see Figure 6.7. Thus the T17-Myc basic region does not appear to be detrimental to *in vitro* binding of oligonucleotides containing the consensus Myc/Max E-box motif.

6.3.4 The T17 C-terminal domain in conjunction with Max, transactivates a reporter gene construct in yeast

The ability of the T17 C-terminus basic region HLH-LZ domains to dimerize with Max and to bind a consensus motif containing the core CACGTG was confirmed by the yeast assay. This assay demonstrated that the basic region mutation does not prevent transactivation from a reporter construct, indeed the T17 mutant maintains the ability to recognise the reporter and activates transcription as efficiently as the wild type, see Figure 6.8.

```
         M   S   D   N   D   D   I   E   V   E   S   D   E   E   Q (15)
        ATG AGC GAT AAC GAT GAC ATC GAG GTG GAG AGC GAC GAA GAG CAA 45
5'-ggaaatg agc gat aac gat g-3'

         P   R   F   Q   S   A   A   D   K   R   A   H   H   N   A (30)
        CCG AGG TTT CAA TCT GCG GCT GAC AAA CGG GCT CAT CAT AAC GCA 90
H                                                           T
M       G                               G           C       T

         L   E   R   K   R   R   D   H   I   K   D   S   F   H   S (45)
        CTG GAA CGA AAA CGT AGG GAC CAC ATC AAA GAC AGC TTT CAC AGT 135


         L   R   D   S   V   P   S   L   Q   G   E   K   A   S   R (60)
        TTG CGG GAC TCG GTC CCA TCA CTC CAA GGA GAG AAG GCA TCC CGG 180
H                   A
M                   A

         A   Q   I   L   D   K   A   T   E   Y   I   Q   Y   M   R (75)
        GCC CAA ATC CTA GAC AAA GCC ACA GAG TAT ATC CAG TAT ATG CGA 225
H
M                           A

         R   K   N   H   T   H   Q   Q   D   I   D   D   L   K   R (90)
        AGG AAA AAC CAC ACA CAC CAG CAA GAT ATC GAT GAC CTC AAG CGG 270
H                                       T   C
M                   G T   G               A   T

         Q   N   A   L   L   E   Q   Q   V   R   A   L   E   K   A (105)
        CAG AAT GCT CTC CTG GAG CAG CAA GTC CGT GCA CTG GAG AAG GCG 315
H                   T
M                   T           A                               A

         R   S   S   A   Q   L   Q   T   N   Y   P   S   S   D   N (120)
        AGG TCG AGT GCC CAA CTG CAG ACC AAC TAC CCC TCC TCA GAC AAC 360
H            A
M         A  A

         S   L   Y   T   N   A   K   G   S   T   I   S   A   F   D (135)
        AGC CTC TAC ACC AAC GCC AAG GGC AGC ACC ATC TCT GCC TTC GAT 405
H
M                                   G

         G   G   S   D   S   S   S   E   S   E   P   E   E   P   Q (150)
        GGA GGC TCA GAC TCC AGC TCG GAG TCG GAG CCC GAA GAG CCC CAA 450
H        G                      A       T       T
M        G   T                  A   A   C       T               G

         S   R   K   K   L   R   M   E   A   S  stop (160)
        AGC AGG AAG AAG CTC CGG ATG GAG GCC AGC TAA   483
                      3'-cc tac ctc cgg tcg att cgg-5'
M                   A
```

Figure 6.2 cDNA sequence of feline *max*.

Figure 6.2. showing the feline *max* nucleotide coding sequence with single letter amino acid code above. Lower case lettering denotes primer sequences. The difference between the feline Max and feline Max9 transcripts is underlined. Bases beneath codons denote the base changes between feline Max and Max (H), and between feline Max and Myn (M). Amino acids 2, 11, 140, 142 and 144 are casein kinase II phosphorylation sites, which can affect DNA binding of Max homo- and Myc/Max heterodimers (Bousset K.(224), Sollenberger K.G. *et al* (225) and references therein).

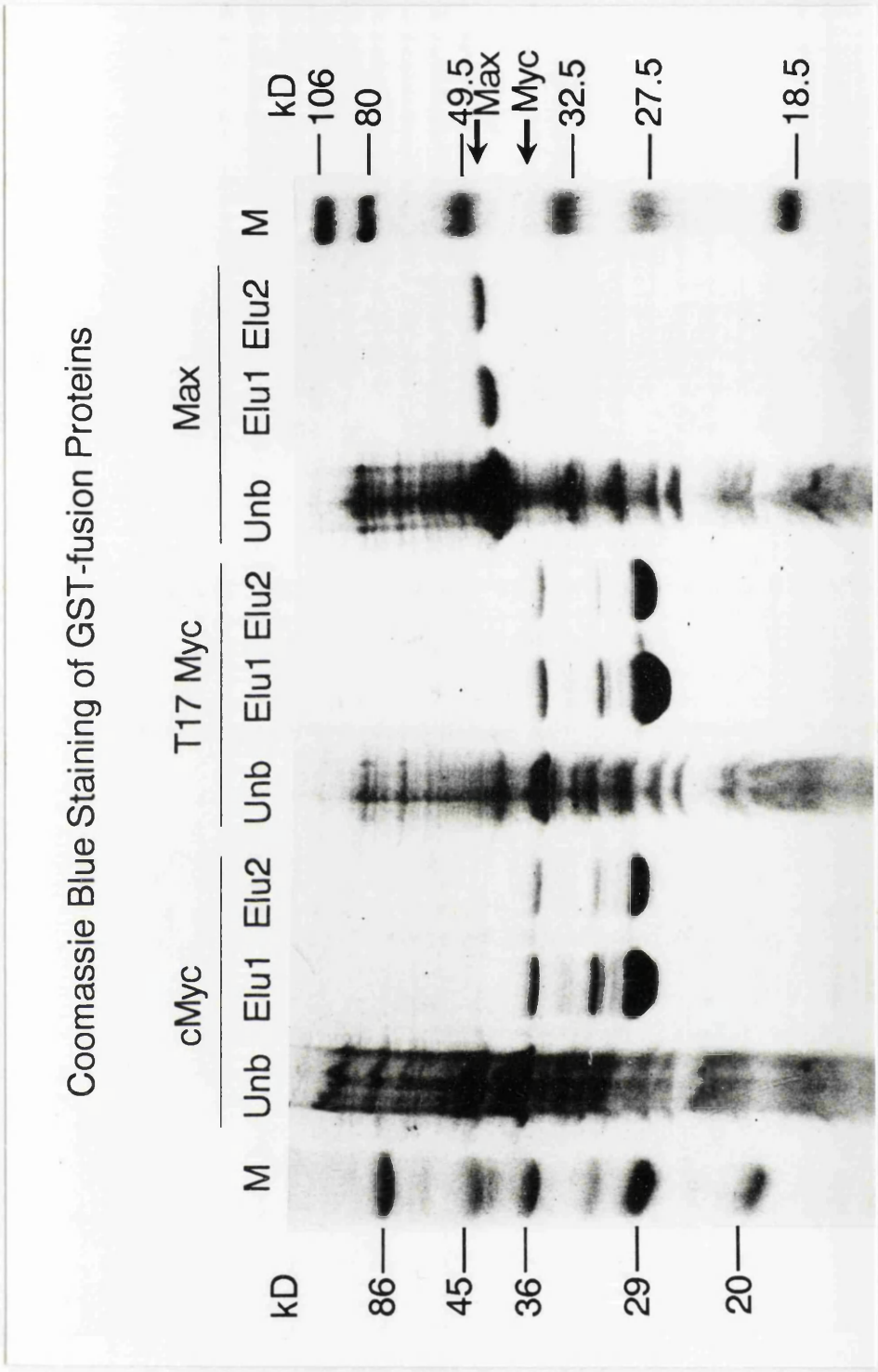


Figure 6.3 Coomassie blue stained SDS-PAGE gel showing glutathione-agarose bead purified recombinant GST-C-terminal Myc and GST-Max proteins.

M, markers; UNB, unbound proteins; ELU1, first eluate from GST-agarose beads; ELU2, second eluate from GST-agarose beads.

This gel demonstrates the relative instability of the GST-Myc constructs compared to GST-Max recombinant, as the large bands at ~28kD in the Myc lanes correspond to GST alone.

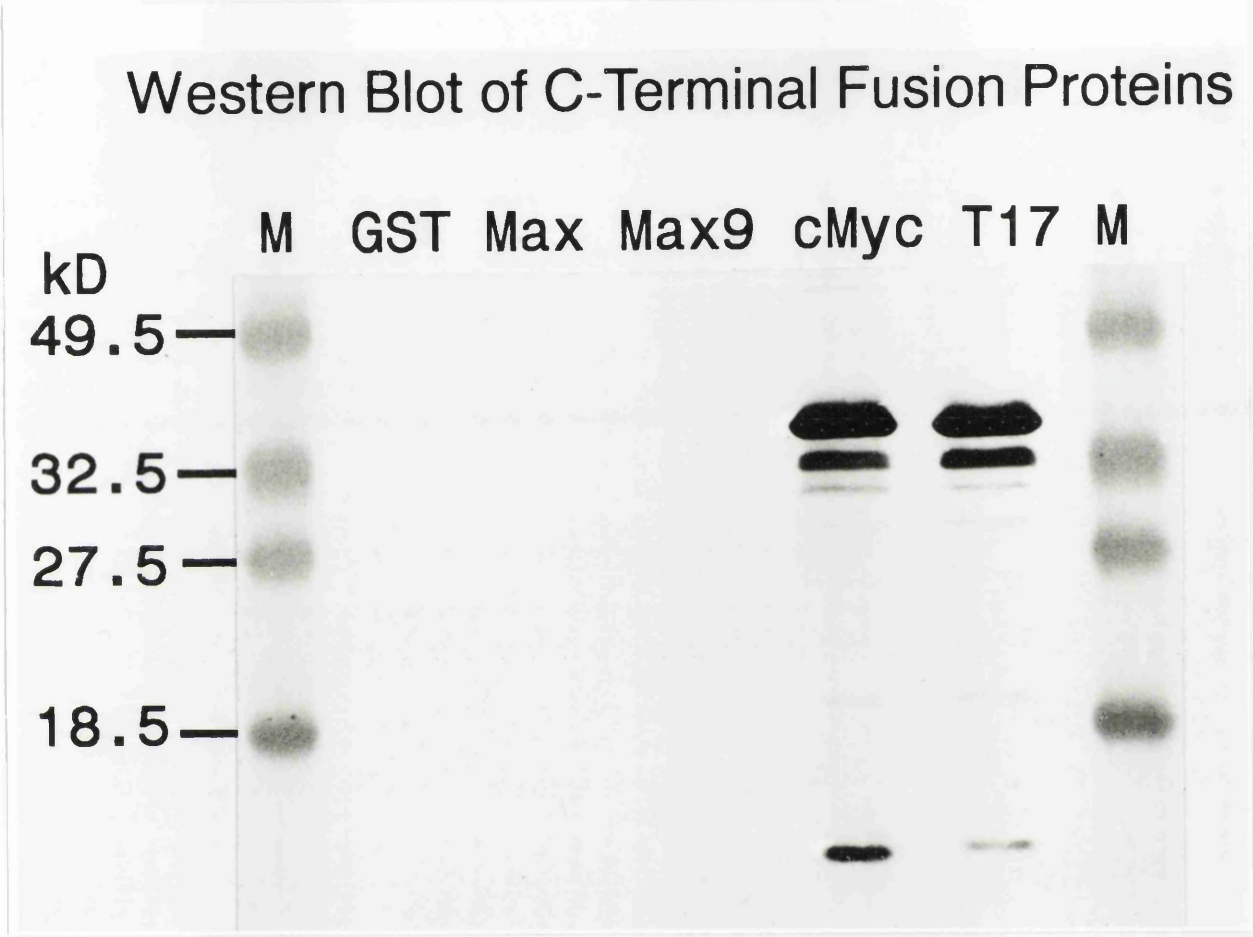


Figure 6.4 Western immunoblot of purified recombinant proteins, using an anti-Myc rabbit polyclonal antiserum.

M, markers.

This figure further demonstrates that the primary antibody does not cross-react with GST or GST-Max.

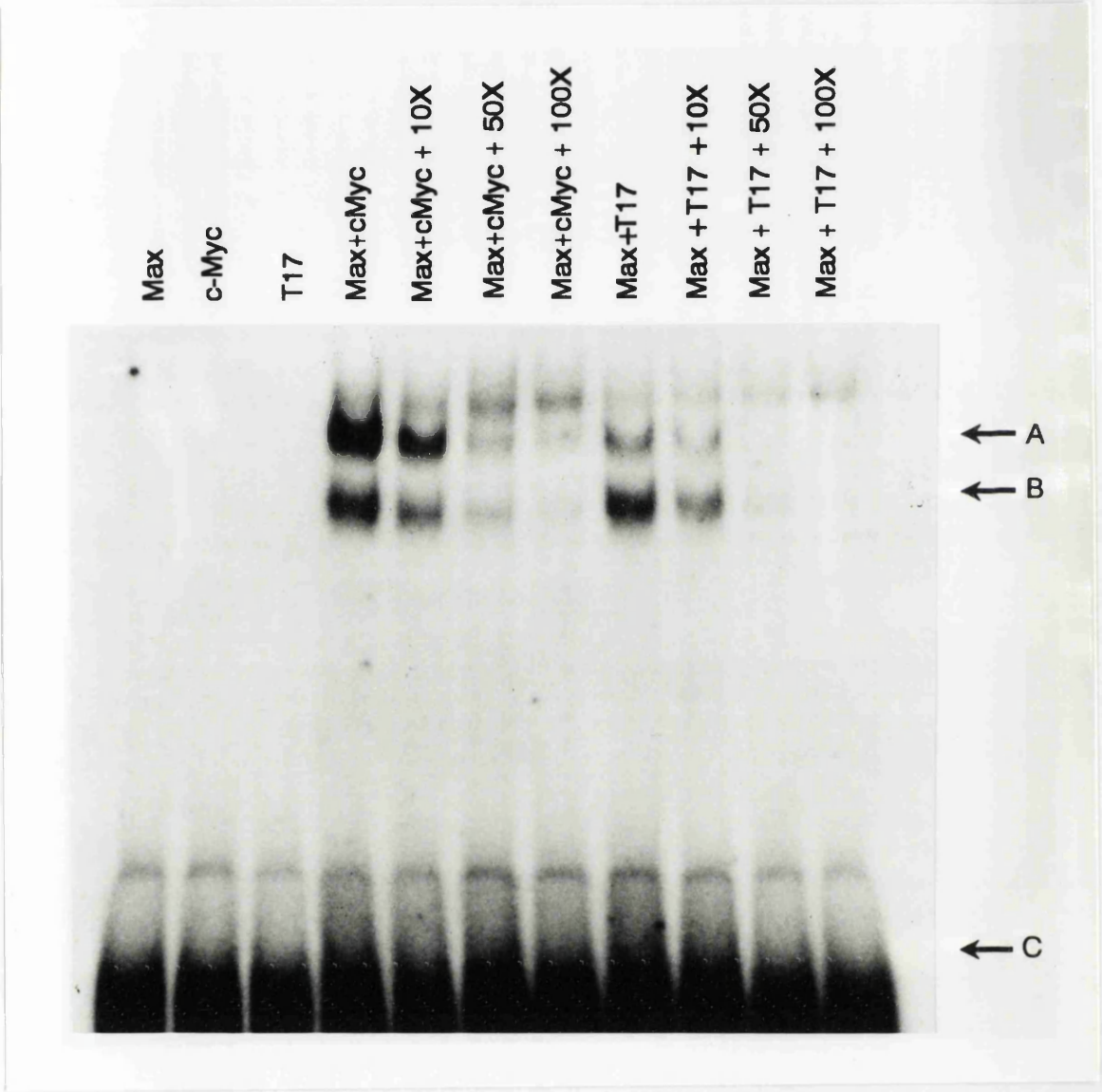


Figure 6.5 EMSA with radiolabelled CM1 oligonucleotide.

Molar ratios of unlabelled competitor oligonucleotide are given above lanes where competitor has been added.

'A', specific band competed with unlabelled CM1

'B', specific band competed with unlabelled CM1

'C', free oligonucleotide

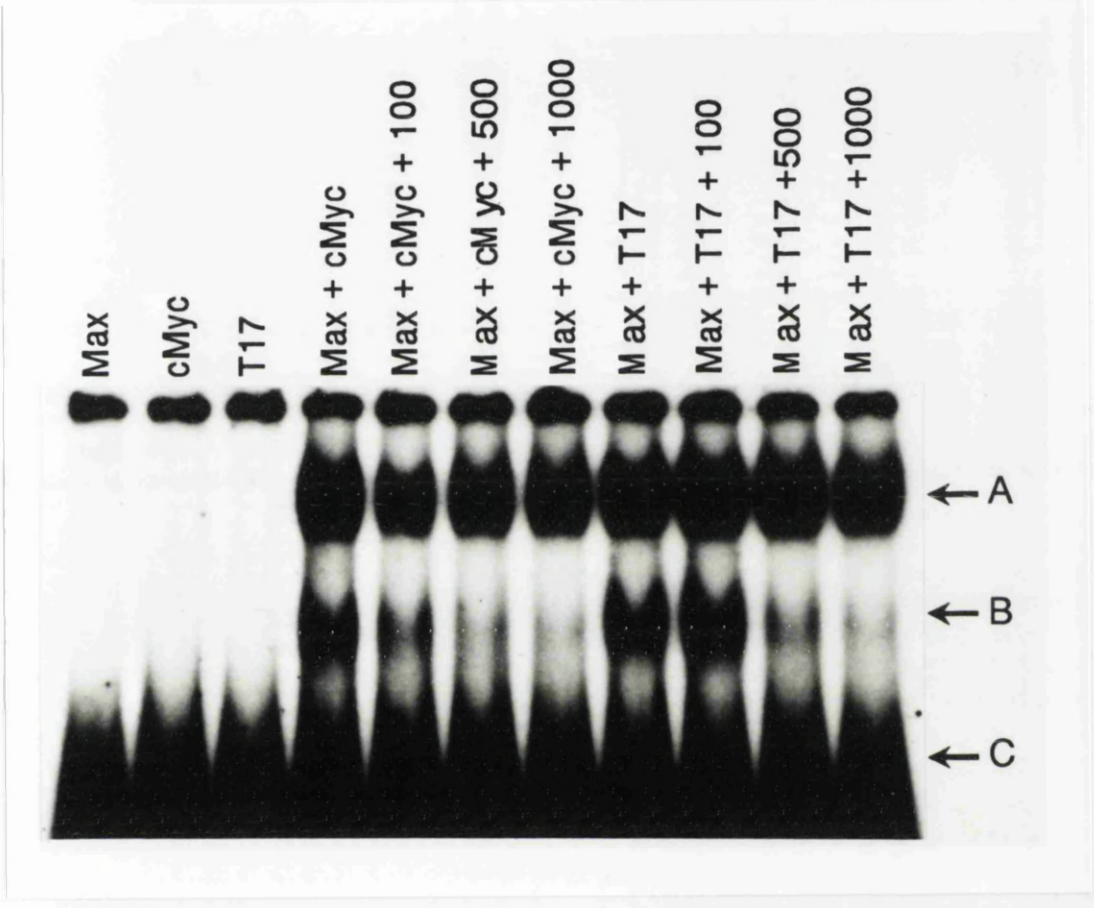


Figure 6.6 EMSA with radiolabelled P2 probe

Molar excess of unlabelled P2 probe is given above lanes where unlabelled competitor has been added.

'A', non-specific binding (see reference 115)

'B', specific binding

'C', free oligonucleotide

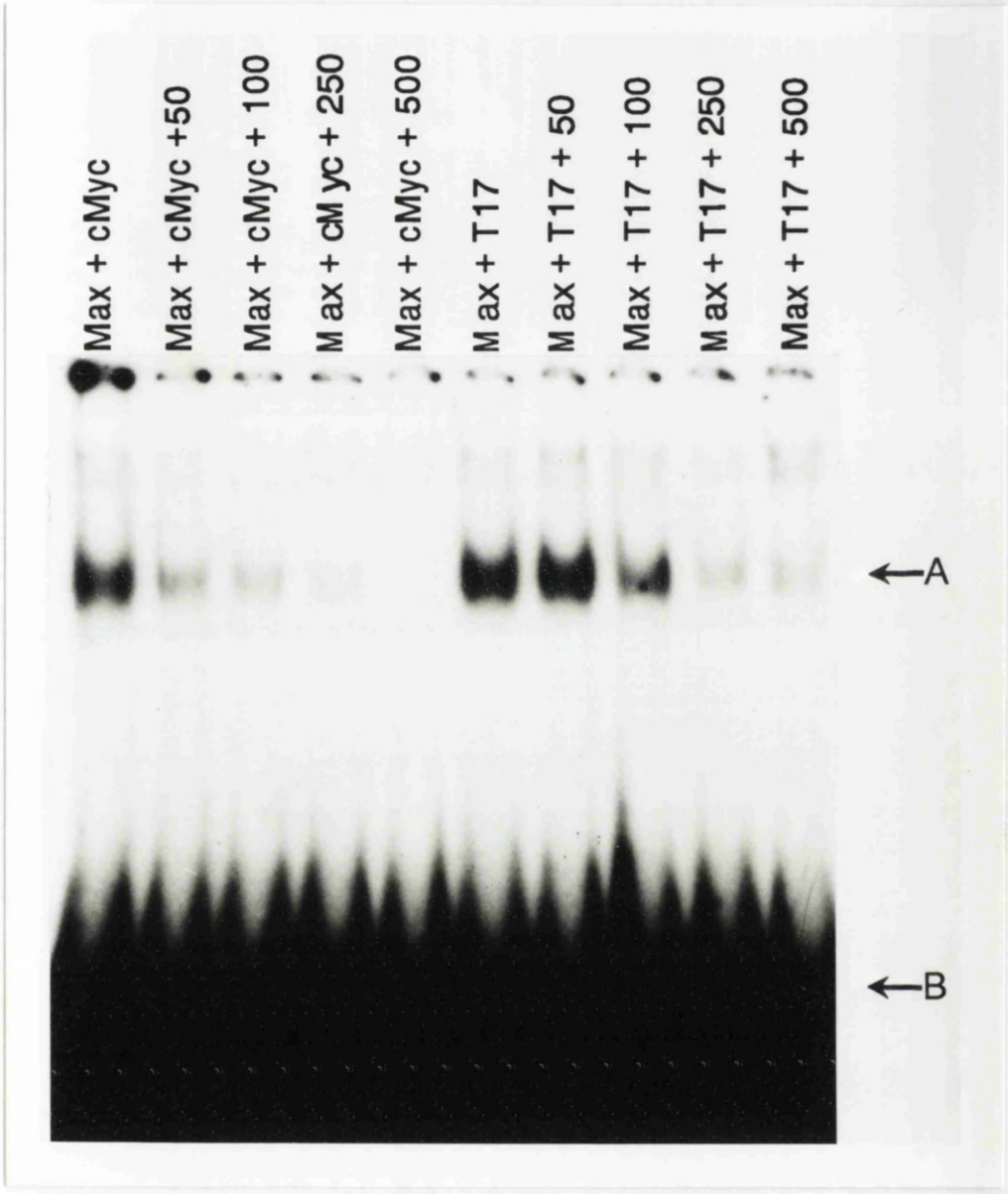


Figure 6.7 Radiolabelled CM1 oligonucleotide, competed with unlabelled P2 oligonucleotide.

Molar excess of unlabelled competitor oligonucleotide is given above lanes where competitor has been added.

'A', competed band

'B', free oligonucleotide

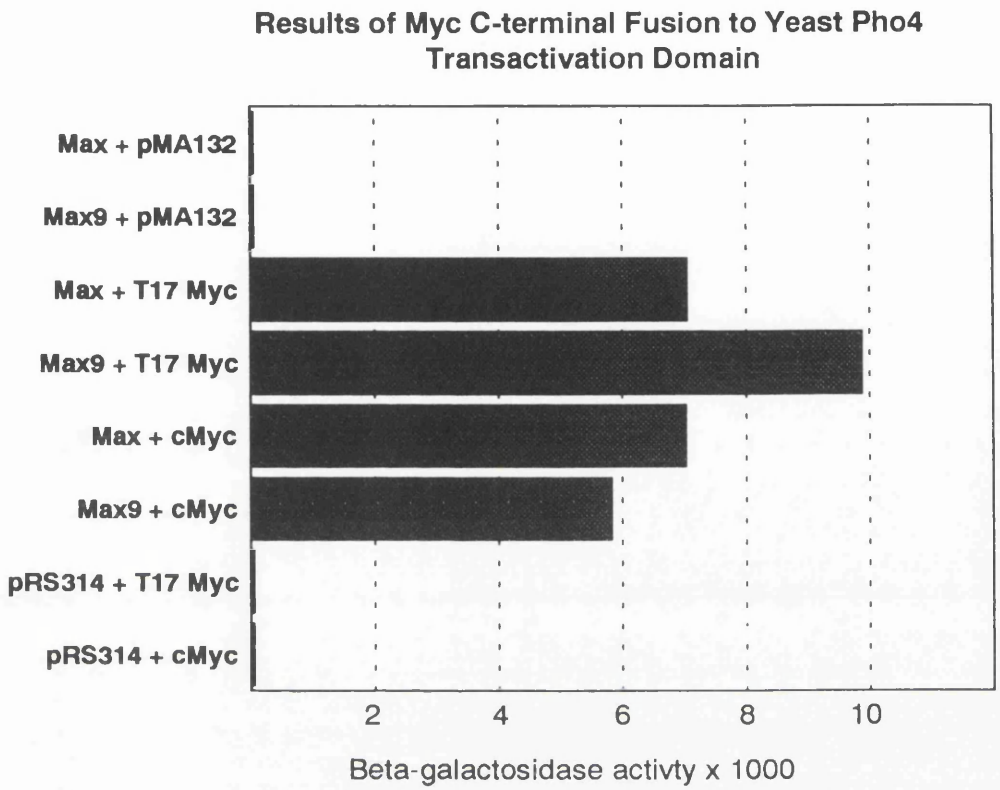


Figure 6.8 This figure demonstrates that there is no defect in the ability of the T17 mutant C-terminal in binding to a reporter construct in yeast. The figure was constructed by averaging the results from two separate experiments.

6.4 Discussion

The mutation at the heart of the T17-Myc basic region might have been expected to alter either the specificity or the avidity of the mutant Myc for its consensus motif. However, previous work has shown that replacement of the equivalent residue, with arginine, in the human N-Myc protein led to the mutant having a broad capacity to bind to several targets, other than the CACGTG consensus (226). This, though interesting, is unlikely to be quite the same for the T17 mutant, as the mutant also has an insertion of a large hydrophobic phenylalanine residue. Another finding of interest is that the leucine which has been replaced in the T17 mutant appears to be necessary to prevent binding to oligonucleotides containing the mutated target sequence 5'-CATATG-3' (226). However, I could find no evidence that GST-T17/Max could bind to an oligo with this core sequence (not shown).

Other research which suggested that bases outside the canonical 5'-CACGTG-3' consensus motif could affect protein-DNA specificity has also been taken into account (221-223). However, no evidence could be found that the T17-Myc mutant reacted any differently from c-Myc in EMSA experiments designed to detect changed specificity.

Figure 6.9 showing a graph of the densitometry results from the EMSA with the CM1 oligo, demonstrates that the avidity of the T17-Myc BR is directly comparable to the avidity of the c-Myc BR. Therefore, the failure of the c/v-Myc chimaera to increase growth rate, and the reduced capacity to cause anchorage independent growth, are unlikely to stem from an inability to bind DNA. Nor does it seem likely that differential on/off kinetics of protein-DNA interactions can account for these changes, as both the affinity and avidity appear to be unchanged for the mutant, thus the mode of action of the T17-Myc C-terminal mutation must be at a more subtle level.

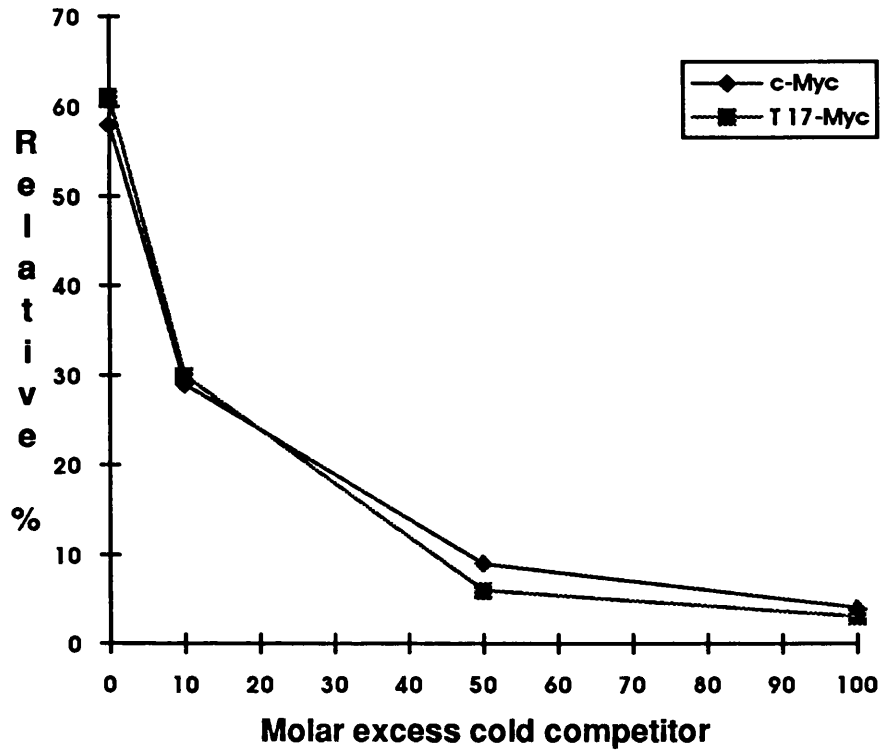


Figure 6.9 This graph was constructed using the densitometry results obtained by densitometry of both specific bands of the CM1 oligonucleotide gel (Figure 6.5) with a Bio-Rad model GS-670 densitometer. In order that like was compared to like, the relative percentage of GST-cMyc or GST-T17Myc was plotted, obtained by totalling the results for all the bands of each protein and dividing by the individual lane.

Further evidence that the T17-Myc BR is as effective as the c-Myc BR in binding DNA when heterodimerised with Max comes from the yeast transactivation assay (figure 6.8). This makes it seem improbable that the observed effects in the CEF assays are due to any defect in DNA binding. Unlike the effects described for the c-MycΔ7 mutant which has a reduced capacity to suppress *myo*D expression and cause fibroblast transformation, but allows transformation of myoblasts (211,217), and only gives half the level of c-Myc/Max

transcription in the same yeast assay as used in this study (176). This makes it seem more likely that the observed deficiencies of the T17-Myc mutant and the c-MycΔ7 mutant are not due to the same mode of action. From the data available the c-MycΔ7 mutant most likely exerts its changed ability to cause transformation due to different kinetics required to transform fibroblasts, where a 50% drop in Myc mediated transcription permits the transformation of myoblasts, but is non-permissive for the transformation of fibroblasts, whereas the T17-Myc mutant appears to be acting in an even more subtle manner. One possible mechanism which could account for the changed transforming abilities of the chimaera carrying the basic region mutation, is that there is an altered ability to bend native DNA. This rather speculative argument is dealt with more fully in the final discussion (chapter 9).

CHAPTER 7

Chapter 7 The N-terminal domain of T17-Myc shows limited ability to activate transcription in a yeast assay and can bind to p107 *in vitro*

Chapter 7 The N-terminal domain of T17-Myc shows limited ability to activate transcription in a yeast assay and can bind to p107 *in vitro*

7.1 Introduction

The N-terminal portion of Myc has been reported to carry a transcriptional activation function (138), and has also been shown to associate with the retinoblastoma protein Rb (227) and the related protein p107 (149). However, where the latter work was able to demonstrate Myc binding to p107 under physiological conditions, there was no detectable binding to Rb under the same conditions (149).

Although a great deal of effort has gone into identifying possible functional domains of Myc, most theories have been extrapolations based on similarity between Myc and known transcription factors. The N-terminal domain was suspected to act as a transcriptional activation domain, from the high numbers of acidic, proline, and glutamine residues which are common in known transcription factor activation domains (134). This observation led to the functional analysis of portions of the Myc N-terminal domain fused to various DNA binding domains, such as the bacterial LexA DNA binding domain (227), and the yeast transcription factor GAL4 DNA binding domain (138). These studies confirmed that the Myc domain would transactivate a reporter construct containing the appropriate target binding site (138,227). To test the ability of the T17-Myc N-terminus to activate transcription in such an assay, the DNA encoding the N-terminal 267 amino acids of feline c-*myc* and 193 amino acids of T17-*myc*, were cloned into the inducible yeast expression vector pV44, giving fusion products with the DNA binding domain of LexA (see Figure 7.1).

As a further test of the capacity of the T17-Myc N-terminal domain to interact with other proteins, GST-Myc constructs were produced using the codons for the N-terminal 254, and 180 amino acids of c-, and T-17 Myc respectively. The protein products were tested for their ability to interact with p107 in an *in vitro* binding assay. This experiment utilised a linked transcription-translation protocol, to produce *in vitro* translated p107 from a partial clone of the human p107 cDNA (160). The rationale for investigating potential binding to p107 comes from evidence that p107 is able to down modulate Myc-mediated transactivation from a reporter construct (227). There is also evidence suggesting that c-Myc mutants derived from lymphomas can resist suppression by p107 (228).

A human p107 gene cDNA clone was also subcloned into the yeast inducible vector pKV701 (177) to investigate the possibility that p107 could suppress Myc activated transcription in the yeast system.

7.2 Materials and methods

7.2.1 Construction of yeast vectors and β-galactosidase assay

Plasmids containing the *myc* N-terminal coding sequences were made by the PCR using primers encoding a BamHI site at the 5' end and a ClaI site at the 3' end (underlined). See figure 7.1. Also encoded by the 3' primer is an in-frame stop codon (shown in bold), as the construct fuses the N-terminus of Myc to the C-terminus of LexA. Primer sequences are as follows:-

5' primer 5'-GAC <u>GGA TCC</u> ATG CCC CTC AAC GTC AGC-3'

3' primer 5'-CGC <u>ATC GAT</u> **TTA** CAC AGA AAC GAC ATC AAT TTC-3'

The human p107 cDNA clone was digested with BamH1 and the p107 fragment purified from a 0.9% TAE agarose gel. pKV701 was digested with Bgl II and treated with phosphatase. Gel purified p107 was then ligated into pKV701 as described in chapter 2, and bacteria were transformed with the ligation mix. Clones were subsequently identified by mini-prep of plasmid DNA and restriction digest with Cla1. Clones with p107 in the correct orientation produced bands of 5.5kb and 4.7kb, whereas the incorrect orientation gave bands of 5.5kb, 2.8kb, and 2.0kb. Large scale plasmid DNA preps were made of appropriate plasmids.

7.2.2 Construction of GST-Myc N-terminal proteins

Myc N-terminal fusion proteins were constructed by the PCR using primers which encode a BamH1 site in the 5' primer and an EcoR1 site in the 3' primer. PCR reactions were carried out as described in materials and methods. Primer sequences were:-

5' primer 5'-GAC GGA TCC ATG CCC CTC AAC GTC AGC-3'

3' primer 5'-GAC GAA TTC TCT TCC TCA GAG TCG CTG-3'

These gave fusion products consisting of 254 amino acids of c-Myc and 180 amino acids of T17-Myc.

7.2.3 *in-vitro* translation of p107

1µg of the human p107 clone (160) was *in vitro* translated using a linked transcription-translation protocol with the TnT coupled wheat germ extract system (Promega). This system allows simple incorporation of ^{35}S-labelled methionine (Amersham. Cat. No. SJ1015), or other amino acids, into translated proteins and includes specific RNA polymerase. For the purpose of transcribing p107, the T3 polymerase was required.

7.2.4 Western immunoblot of GST-Myc

Western immunoblot of N-terminal Myc-GST was carried out essentially as previously described in section 6.2.4.

7.2.5 *in vitro* Myc-p107 binding assay

This procedure was adapted from previously published work using *in vitro* translated proteins binding to GST-fusion protein (229). Before binding assays were carried out the *in vitro* translated p107 protein was pre-cleared of non-specific binding moieties, by incubation with glutathione-agarose beads. These beads were then spun out by centrifugation in 1.5ml eppendorf tubes, at 2000rpm in a microfuge, and the supernatant retained for use in binding reactions.

Myc-GST was initially eluted from glutathione-agarose beads then dialysed overnight against 1M TRIS pH7.5. Dialysed Myc-GST was then concentrated and equivalent amounts of protein were rebound to fresh glutathione-agarose beads, the re-bound fusion protein was made into a slurry at 1:1 with binding buffer (20mM Tris; 150mM NaCl; 0.2% Triton-X). 50µl of the slurry was incubated with 25µl of the cleared *in vitro* translation reaction at 4°C for 20 minutes, with occasional gentle agitation.

Following incubation reactions were washed four times with 300-500µl of binding buffer. Beads were then concentrated by centrifugation at 2000rpm in a microfuge, bound proteins were then eluted by boiling the beads in 25µl of loading buffer. Eluted protein was loaded directly onto a 10% SDS-PAGE gel and electrophoresed, the gel was then fixed by

submerging in destain for 15 minutes. The gel was then dried and exposed overnight, or longer, to Kodak X-ray film.

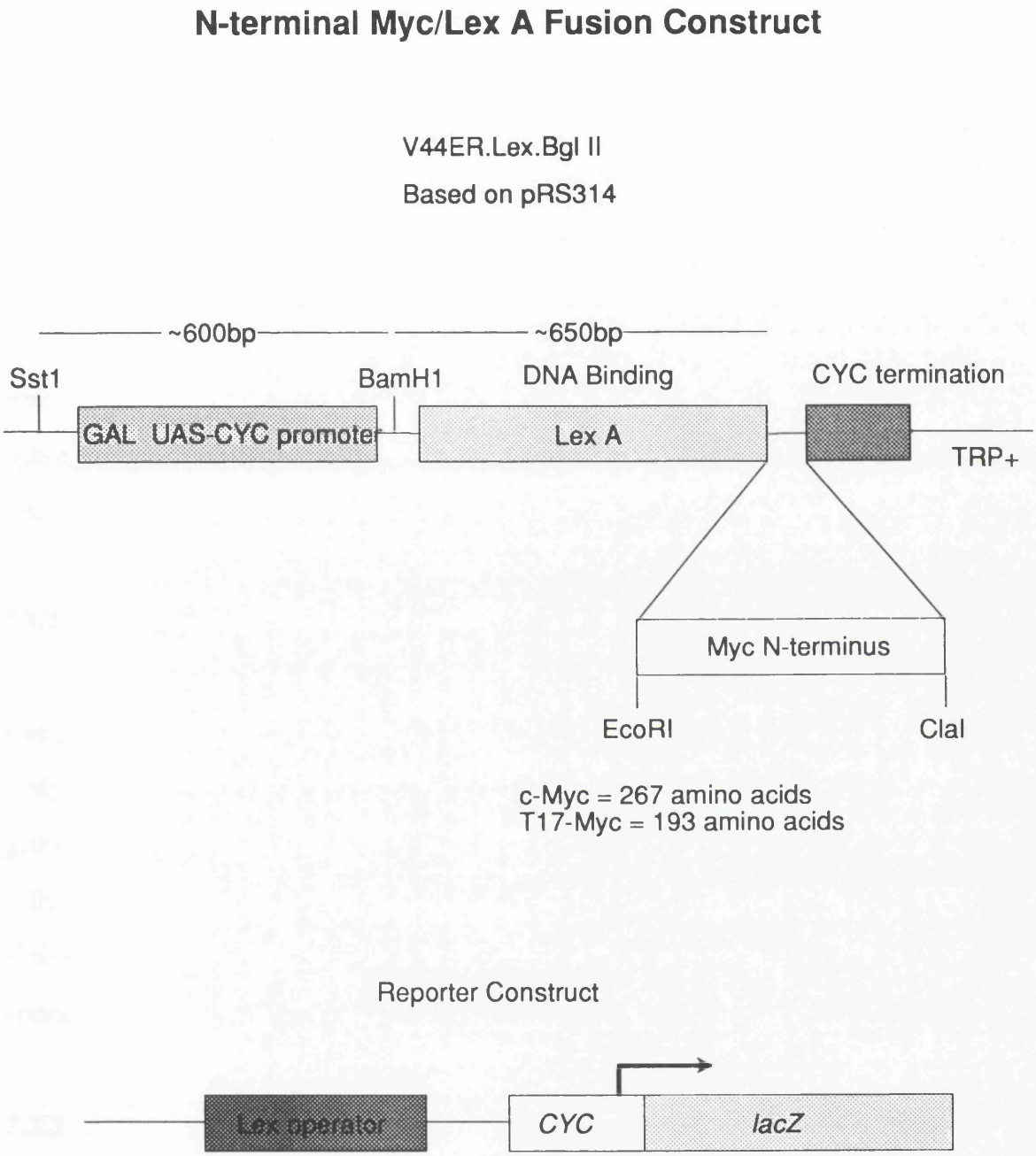


Figure 7.1 Myc N-terminal transactivation constructs used in yeast assays.

7.3 Results

7.3.1 The feline c-Myc N-terminal domain transactivates in a yeast assay, whereas the T17-Myc N-terminus shows limited activity in the same assay

The results of using the T17-Myc N-terminus in a yeast transcriptional activation assay are demonstrated in Figure 7.2. This figure shows that there is activation of the reporter construct, suggesting that the T17-Myc N-terminus is not transcriptionally redundant. Confirmation that the fusion constructs were translated correctly is provided by western immunoblot of yeast cultures, using a rabbit polyclonal anti-Myc serum (Figure 7.3). This figure also indicates that the T17-Myc N-terminus may well be more stable in yeast, as equivalent cell numbers show greater levels of v-Myc protein.

7.3.2 p107 was not detected in cells carrying the expression plasmid

Use of the inducible p107 plasmid failed to show any effect on transcription mediated by the c-Myc N-terminus (not shown). However, this seems likely to be due to some failure in getting expression of p107 from the expression vector, as western immunoblot of induced cells using an anti-p107 antibody (gift of Dr. L. Allen, Department of Biochemistry, University of Glasgow) did not detect the protein, despite the vector having mapped correctly by restriction enzyme analysis.

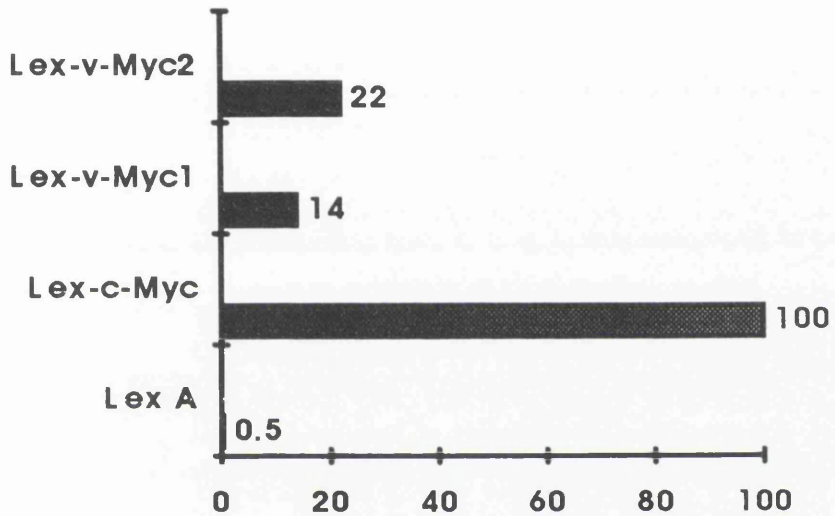
7.3.3 Western immunoblot of GST-Myc constructs

To ensure that the bacterially produced GST fusion proteins were correctly translated, western immunobloting was carried out on glutathione-agarose purified protein. The result of this experiment demonstrates that the recombinant Myc protein constructs used for the

Myc-p107 binding experiments are valid translations of the Myc protein, see Figure 7.4. The protein products obtained, consistently showed a ratio of at least 2.5:1, of T17 to c-Myc in the yield obtained from a given volume of bacterial culture. However, it is not clear whether this was a reflection of an increase in stability of this portion of Myc in bacterial cells, or whether plasmid copy number in the clones selected played a role.

7.3.4 The T17-Myc N-terminal domain can bind to p107 *in vitro*

As there are still few known elements of the transcriptional machinery which interact directly with Myc (other than Max), p107 is a promising candidate as a modulator of Myc function (149,162). Therefore, any possible interaction with T17-Myc could provide valuable insights into the method of action of the T17-Myc mutant in leukaemogenesis. The rather surprising result of this set of experiments is that the T17-Myc N-terminus is capable of interacting with 107 *in vitro* (Figure 7.5).



β-Galactosidase assay.

Y-axis gives percentage activity of v-Myc clones compared to c-Myc as 100% (liquid β-galactosidase assay was carried out as detailed in Section 6.2.2).

Figure 7.2 LexA-Myc-N-terminal fusion β-galactosidase assay in yeast

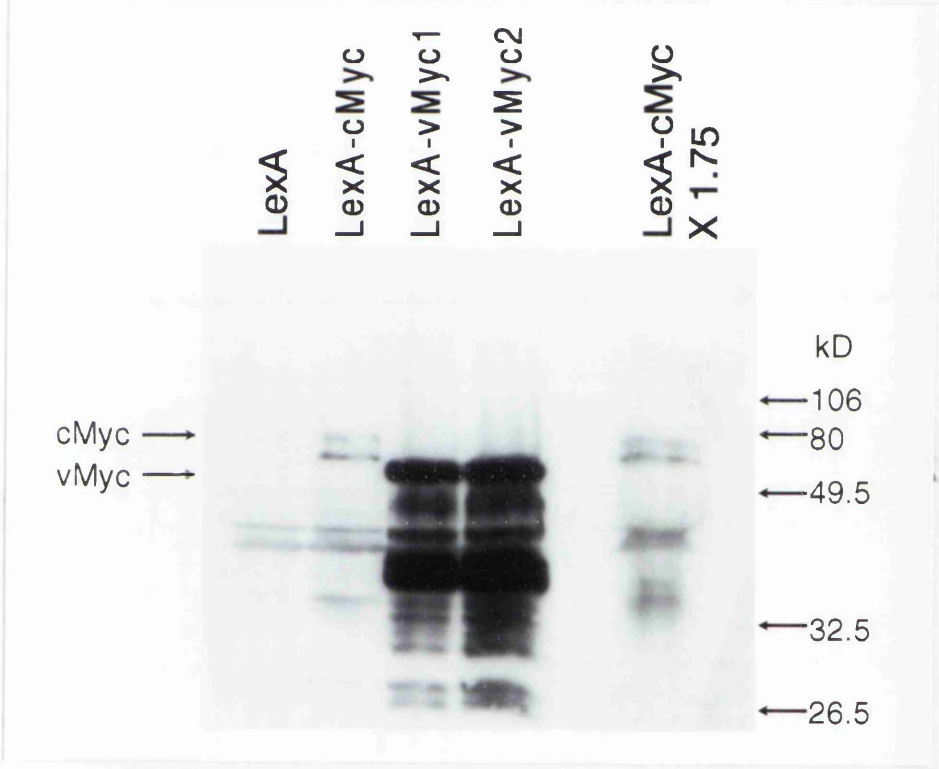


Figure 7.3 Western immunoblot of yeast cultures using an anti-Myc rabbit polyclonal antiserum, demonstrating that the fusion constructs are translated correctly. Equivalent numbers of yeast cells have been run in each lane, except c-Myc 1.75 where 1.75 times the number of cells were used.

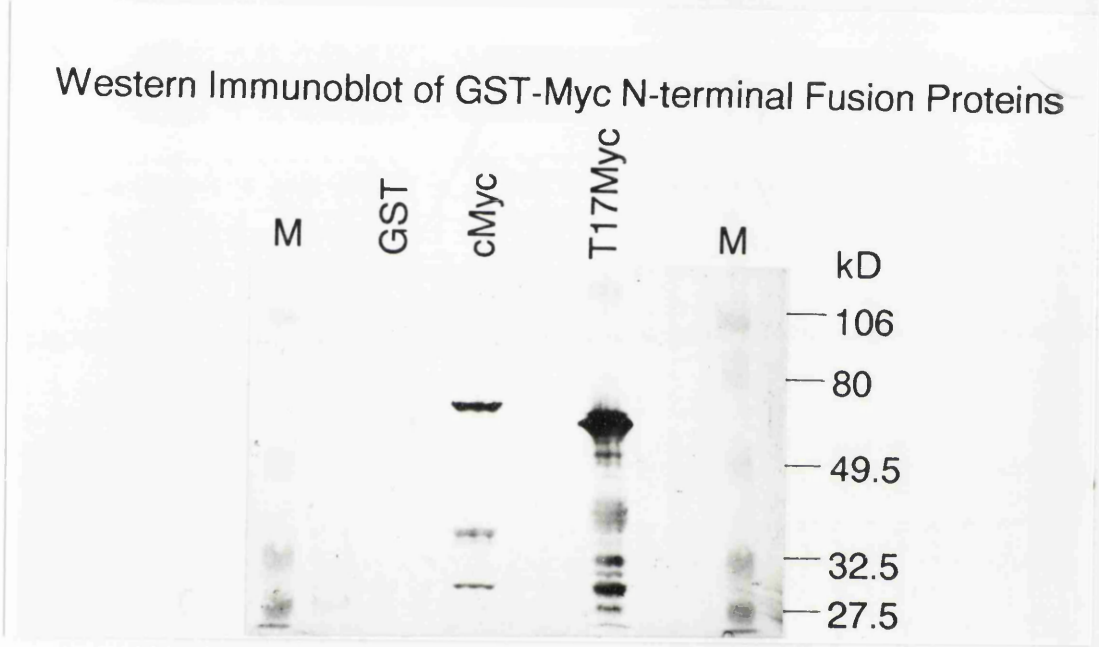


Figure 7.4 Western immunoblot of GST-Myc N-terminal fusion proteins

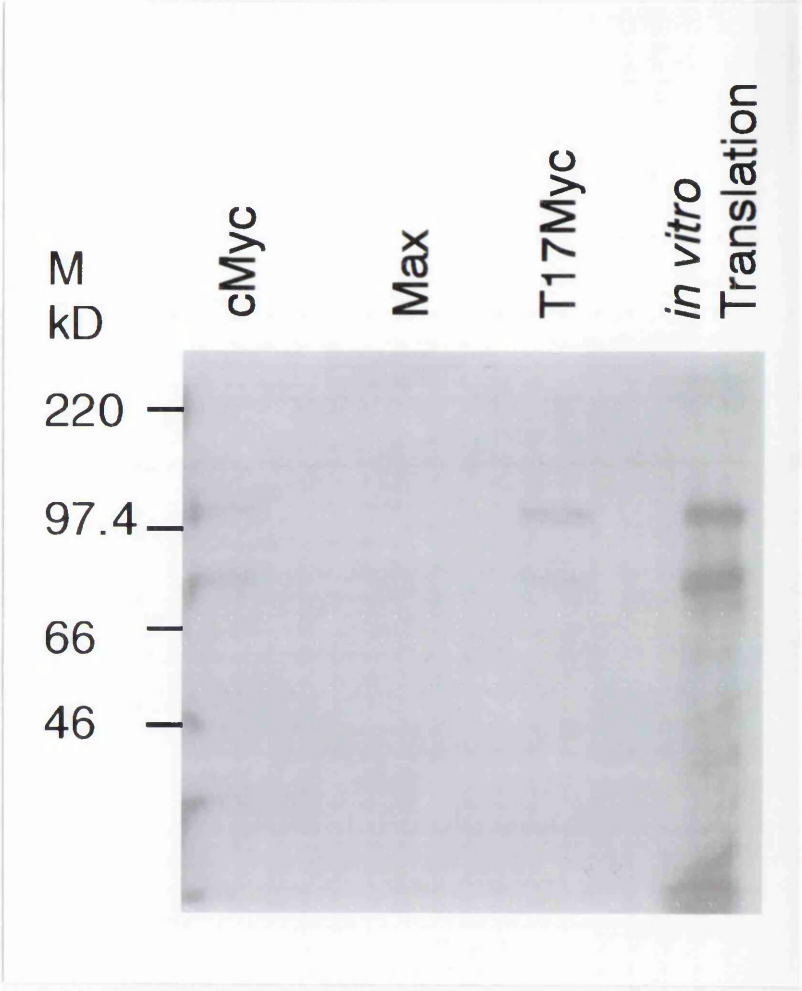


Figure 7.5 *in vitro* binding assay with GST-Myc N-terminal proteins and *in vitro* translated p107.

Two bands are visible in the Myc lanes, these correspond to a molecular weight of ~100kD and ~80kD, both bands are large enough to contain the pocket domain which mediates binding to other proteins (160).

7.4 Discussion

The finding that the T17-Myc N-terminus can activate transcription in a yeast assay, albeit at a reduced level, is perhaps surprising, as this domain conferred no apparent phenotype in the CEF assays (Chapters 4 and 5). This yeast assay demonstrates that the T17-Myc mutant does have activity other than DNA binding in association with Max. Further evidence of activity is that the N-terminus retains the ability to interact with p107 *in vitro*.

A great deal of work has focused on the abilty of the N-terminus of Myc to promote transcription. Allied to the transcription work has been the desire to understand how mutations in the N-terminus can suppress or enhance the ability of Myc to cause transformation. Recently there have been several reports that mutations in the coding region of c-*myc* occur at a high incidence in B cell lymphomas of both humans (230,231) and mice (232). Many of the reported mutations have been shown to affect the second exon of c-*myc* (230,233,234). Close examination of these reports show that a large percentage of reported mutations are within the regions deleted in the T17-*myc* mutant. In one report detailing 30 Burkitt's lymphomas carrying c-*myc* mutations, 23 of the lymphomas had c-*myc* mutations within the regions deleted in T17-*myc* (232), while in other reports 50% (230), and 76% (234) of cases had mutations in the same region.

Further evidence that these mutations could be important was demonstrated by use of GAL4/c-Myc fusion proteins. Fusion constructs were shown to decrease activity two to five fold, from a luciferase reporter plasmid when mutations found in the lymphoma-derived Myc proteins were present (234). The mutations used also included replacement of Thr-58 and Ser-62 with aspartic acid to mimic constitutive phosphorylation, but these also led to reduced reporter construct activity compared to a wild type Myc (234). This finding is in opposition to the idea that phosphorylation of these sites activates transcription (207,235).

Phosphorylation leading to transactivation by a GAL4/c-Myc chimaera has been shown to be cell cycle regulated, with increased activity at the S to G_2 transition (235). One proposal is that phosphorylation of the transactivation domain activates genes involved in cell cycle progression. Loss of phosphorylation sites would free cells from this control (234). These data suggest that an increase in transcriptional activation may not be the method by which Myc acts in transformation of lymphocytes, and this may help to explain the conundrum of T17-*myc*.

The ability of a fusion protein to bind other proteins *in vitro* is not always a good indicator of *in vivo* interaction. The original observation that c-Myc could interact with pRb was made using a GST-Myc N-terminal fusion construct which bound to pRb present in cell lysates (236). However, further work with a fusion construct using the N-terminal 210 amino acids of c-Myc to screen a cDNA library from a B cell lymphoma cell line, identified p107 as a candidate N-terminal binding partner (149). Analysis of immunoprecipitates of Myc were shown to contain p107, and immunoprecipitates of p107 were found to contain Myc, but no Myc-pRb interactions could be detected by immunoprecipitation (149). Further, it was reported that amino acids 41 to 103 of Myc were important for transcriptional suppression by p107, and from this it was concluded that this was the region to which p107 binds (149). Nevertheless, the data from the *in vitro* binding assays shows that T17-Myc is capable of interacting with p107, while Max does not, further the evidence given that amino acids 41 to 103 of Myc mediate binding is at present circumstantial. These results demonstrate that T17-Myc does have the ability to interact, at least *in vitro*, with some members of the transcriptional control machinery.

Together with the data showing that B cell mutants have a reduced capacity to activate transcription, these results suggests that T17-Myc is not unique in its inability to activate transcription, although it may perhaps be unique in the size of deletions carried. Therefore,

the mode of action of T17-Myc in transformation may mimic that of mutated Myc in Burkitt's lymphomas, where reduced transactivation has been proposed to provide a growth advantage (234). What is also interesting is that transcriptional activation which can be measured from lymphoma derived mutants, is refractory to p107 induced suppression (228). One could hypothesise that amino acids 41 to 103 of Myc are important for p107-mediated transcriptional repression (149), not because they directly interact with p107, but rather because they interact with some additional protein required for transcriptional repression by p107. Such an hypothesis would account for the ability of T17-Myc and other lymphoma-derived Myc mutants to associate with p107 (149).

The data also suggest the possibility of a threshold effect, whereby lymphocytes could be sensitive to a lower level of Myc mediated transcription than fibroblasts. Yet another possibility is that lymphocyte-specific factors, which are not available in the systems used to detect Myc mediated transcription, play a role in transcription in lymphocytes. This latter hypothesis certainly seems probable when one tries to account for the differing abilities of various Myc mutants to transform different cell types (see Table 4.1).

CHAPTER 8

Chapter 8 Transcription of putative Myc regulated and Myc regulating genes in feline leukaemic cell lines

Chapter 8 Transcription of putative Myc regulated and Myc regulating genes in feline leukaemic cell lines

8.1 Introduction

Although there have been many claims in the literature of genes which are regulated by c-Myc, there is still a paucity of firm evidence for direct effects. However, at least two genes have been shown to be upregulated in response to Myc induction, viz. ornithine decarboxylase (169,237) and prothymosin-α (166,238), while two other BR-HLH-LZ proteins, known as Mad (123) and Mxi1 (122), have been suggested to modulate the activity of c-Myc via interaction with Max.

Ornithine decarboxylase (ODC) is the first and rate limiting enzyme in the polyamine biosynthesis pathway, and has been shown to be required for entry into and progression through the cell cycle (239). ODC is activated as an "early" gene in G1, however this activation is after induction of "immediate-early" genes such as *fos*, *jun* and *myc* (240).

There is now a growing body of evidence that ODC is transcriptionally activated by c-Myc (167,237). Evidence to this effect includes the use of conditionally active c-Myc-oestrogen receptor constructs, which when transfected into cells show an increase of ODC mRNA and enzymatic activity, dependent on addition of oestrodiol (168). Other work used constructs utilising ODC promoter regions linked to a heterologous reporter gene and showed that c-Myc could transactivate these constructs, without necessarily binding to Max, as expression was reported with mutants which carried deletions or point mutations in the LZ domain (169). This latter finding suggested that c-Myc could regulate transcription via interaction with other proteins in the absence of Max, but has since been contradicted by another group, who suggested that the heterodimer Max is indeed required for activation of the

ODC gene (241). One study focused on CACGTG motifs at -491 to -474 to the transcription start site as the activity regulating site (167), while other work led to the conclusion that the c-Myc binding site(s) is actually situated in the first intron of the ODC gene (169,241). Therefore, although the balance of evidence favours a c-Myc/Max binding site in the first intron of the ODC gene, there is still debate as to the mechanism of c-Myc regulation.

Another putative c-Myc regulated gene is prothymosin-α, but unlike ODC, the function of the prothymosin-α gene product is unknown. Originally presumed to be a thymus specific hormone, the prothymosin-α gene product is now known to be widely distributed in different cell types, and to have an apparent nuclear localisation (242). It is also possible that prothymosin-α plays a role in cell division, as antisense oligomers to prothymosin-α can inhibit myeloma cell division (243), while degradation of the intracellular oligomers accompanied a resumption of cell division. Evidence for a direct role in transcription by c-Myc came initially from subtractive hybridisation using oestrogen stimulated cells expressing an oestrogen receptor-Myc chimaeric protein. This experiment showed the cDNA of prothymosin-α to be over-represented after stimulation of cells with oestrogen (166). Further, albeit circumstantial, evidence for an involvement of c-Myc in transcriptional upregulation of prothymosin-α, came also from a subtractive hybridisation experiment. However, in this latter experiment subtraction was carried out with normal human mucosa and human colon cancer, and screening of the resulting cDNA library. This study showed that there was a statistically significant correlation between the levels of c-myc RNA and the levels of prothymosin-α RNA (238). Again, as with ODC, Myc/Max heterodimers appear to act as enhancers by binding to the CACGTG motif within the first intron of the prothymosin-α gene (244).

The study of transcriptional regulation by c-Myc has yielded conflicting results, and the addition of the Mad and Mxi1 genes further complicated the picture (122,123). Both of these proteins are thought to downregulate Myc activation via heterodimerization with Max. The proposed mechanism of negative regulation is that these novel proteins sequester Max which is required by Myc for binding to the Myc/Max recognition sequence and hence they compete with transcriptionally active Myc-Max heterodimers for target binding sites (122,123).

To examine possible correlations between the levels of c-Myc and T17-Myc proteins and the transcription of ODC and prothymosin-α, northern blot analysis was carried out on feline lymphoma cell lines which overexpress Myc. Further northern blot analysis was undertaken to estimate levels of the *mad* and *mxi*1 transcripts, as well as *max*, in an attempt to find differences between the T17 cell line and the c-Myc expressing cell lines which might help explain the mode of action of the T17-Myc mutant.

8.2 Materials and Methods

8.2.1 Myc expressing feline leukaemic cell lines (RNA extraction)

Cell lines used in this experiment were the feline c-Myc expressing cell line 3201 which expresses an abnormal allele (178), v-Myc expressing F422 and T17, as well as the control fibroblast line AH927. Total RNA was extracted using the RNazol method (Biogenesis), and northern blot analysis was carried out as described in Materials and Methods 2.2.4.1

8.2.2 Cloning and sequencing of the feline prothymosin-α cDNA

To probe for the prothymosin-α transcript, the cDNA for the coding sequence of the feline homologue was first cloned. This was achieved by designing primers which conformed to conserved sequences, between human, rat, and mouse transcripts (245-247). These primer sequences were:-

5' Primer 5'-CGG CGT GCC CCA CCA TG-3'

3' Primer 5'-GCT GTC TAG TCA TCC TC-3'

3.5μg of total RNA isolated from normal feline thymus using the RNazol method (Biogenesis), was subjected to first strand cDNA synthesis using a commercial kit (Pharmacia) and including 175pmol of the 3' primer. 160pmol of the 5' primer was added to the reaction for the subsequent polymerase chain reaction step. The conditions for the 30 cycles of PCR reaction were denaturation at 94°C for one minute, annealing at 55°C for one minute and elongation at 72°C for one minute. A major reaction product of approximately 350bp was observed, when the reactions were subjected to gel electrophoresis on a 6% polyacrylamide gel. These fragments were cloned into the PCR cloning vector pCR II using the TA kit (Stratagene). Each clone was identified by restriction enzyme digest using EcoRI, followed by analysis on a 6% polyacrylamide gel. Four clones carrying an insert of the correct size were sequenced on both strands using a long read Sequitherm cycle sequencing kit (Epicentre Technologies), with an infrared labelled M13 forward or M13 reverse primer (Li-Cor) and sequenced on a Li-Cor model 4000 automated sequencer. One clone showed a single base change compared to the other three, which could have been due to a misincorporation by the *Taq* DNA polymerase.

8.2.3 Preparation of probes and northern blot analysis

Probes used in this set of experiments were; the human ODC cDNA (248), feline specific prothymosin-α (see above); feline specific *max* probe (see 6.2.1); human *mad* cDNA (123); and the human *mxi*1 cDNA (122). These probes were labelled with ^{32}P by use of the random prime kit (Boehringer). Northern analysis was carried out as detailed in 2.2.4.1.

8.3 Results

8.3.1 ODC expression in Myc overexpressing cells compared to a control fibroblast cell line

Densitometry carried out on the X-ray photographic film of the northern blots showed that there is a small increase in the quantity of ODC mRNA in the Myc expressing cell lines compared to the GAPDH standard (Figure 8.1). Although there was no dramatic increase in the levels of mRNA, there was certainly no obvious deficit in the T17 cell line.

8.3.2 Prothymosin-α expression in Myc Overexpressing Cell Lines, Compared to a control fibroblast cell line

Comparison of the levels of GAPDH and prothymosin-α mRNA show a markedly high level in the Myc expressing cell lines compared to the control cell line (Figure 8.2). Again the T17 cell line had high levels of transcripts, indeed the T17 cell line produced detectably more mRNA than either the other v-Myc F422, or the rearranged c-Myc 3201 cells. The sequence of the feline prothymosin cDNA coding sequence is given in figure 8.3.

```
  1 ATGTCAGACGCGGCCGTGGACACCAGCTCCGAGATCACCACCAAGGACTT 50
              .         .         .         .         .

 51 AAAGGAGAAGAAGGAAGTTGTGGAGGAGGCGGAGAATGGAAGAGACGCCC 100
              .         .         .         .         .

101 CTGCTAATGGGAACGCTAATGAGGAAAATGGGGAGCAGGAGGCTGACAAT 150
              .         .         .         .         .

151 GAGGTAGATGAAGAAGAGGAAGAAGGGGGAGAGGAAGAAGAGGAGGAGGA 200
              .         .         .         .         .

201 GGAAGGTGATGGGGAGGAAGAGGATGGAGACGAAGATGAGGAGGCTGAGG 250
              .         .         .         .         .

251 CAGCTACGGGCAAACGGGCAGCTGAAGATGATGAGGATGACGATGTCGAC 300
              .         .         .

301 ACCAAGAAGCAGAAGACCGACGAGGATGACTAG 333
```

Figure 8.3 cDNA sequence of the feline prothymosin-α gene coding region.

The feline coding sequence shows 94% similarity to the equivalent human coding sequence.

8.3.3 Max expression in feline lymphoma cells

Levels of *max* have previously been shown to be similar in quiescent, mitogen induced or cycling cells (249). This is borne out in the comparison of *max* mRNA levels in the Myc expressing cell lines which was found to be at similar levels to the control cell line (Figure 8.4).

8.3.4 Mad transcripts are detectable in all cell lines

Transcripts of the *mad* gene were detected in all the cell lines tested. Figure 8.5 demonstrates that the feline transcripts of this gene are in the same size range (~4kb) as those detected for the human gene (250). Again levels of this gene transcript appeared to be at a rather constant level in the cell lines tested. However, this figure also demonstrates that the detected transcripts appear to be degraded, and therefore may be unstable. Alternatively this could be due to use of a cross-species probe which does not hybridise well with the feline transcript, as the control GAPDH probe gives a more well defined band, thus degraded RNA seems like a less likely explanation.

8.3.5 Mxi1 transcripts are detectable in all cell lines tested

Figure 8.6 demonstrates that transcripts encoding *mxi*1, the second of the Max binding partners, are also detectable in the four cell lines. Again, the feline RNA transcript is in the same size range as the human form (2.8 kb) (250). However, the transcript detected gives a more defined band in comparison to the *mad* transcript. Thus, although the *myc* gene in these cell lines is abnormal, the ability of the cells to produce, at least at the mRNA level, elements to control Myc function have not been affected. However, this conclusion does

not cover the possibility that Myc regulation is affected by factors other than Mad and Mxi1 interaction with Max (122,123).

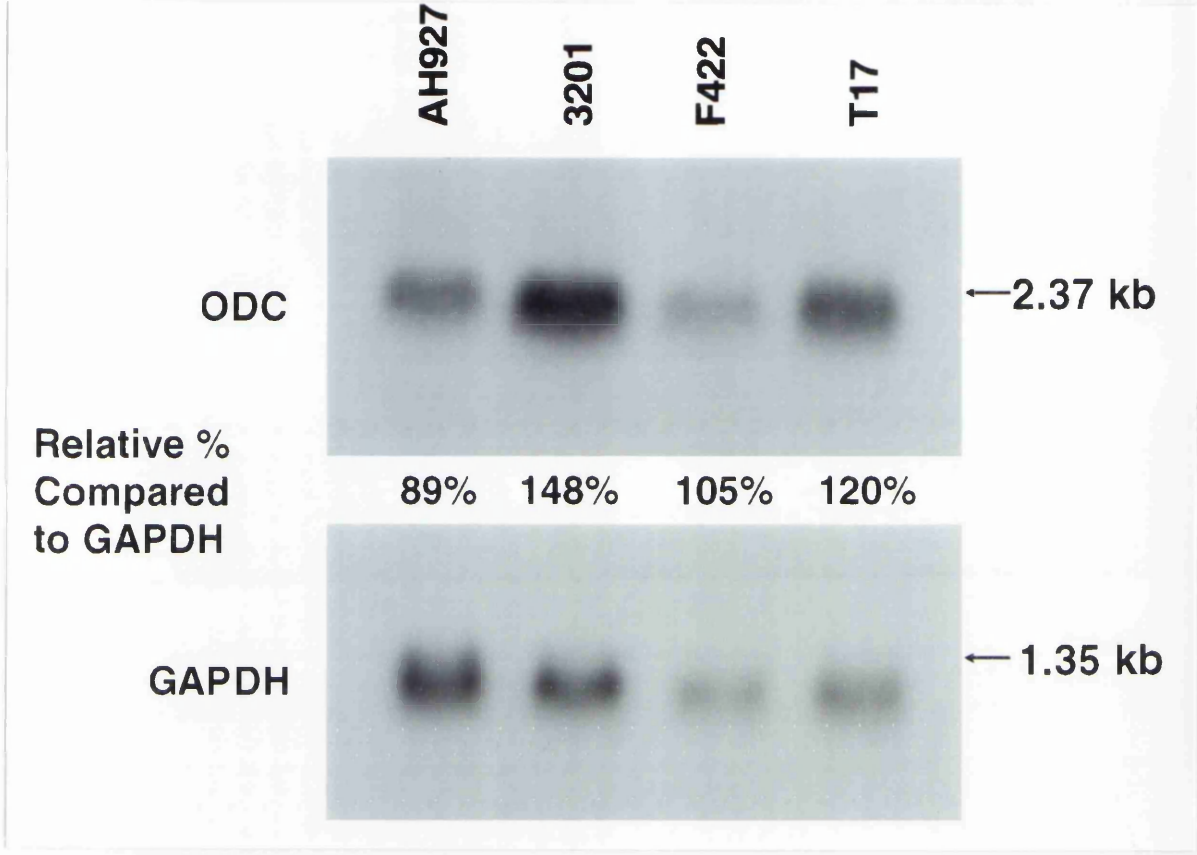


Figure 8.1 Ornithine decarboxylase transcription in lymphoma cell lines. Transcript sizes are ~1.3 kb for GAPDH and ~2.2 kb for ODC. The closest size marker has been shown.

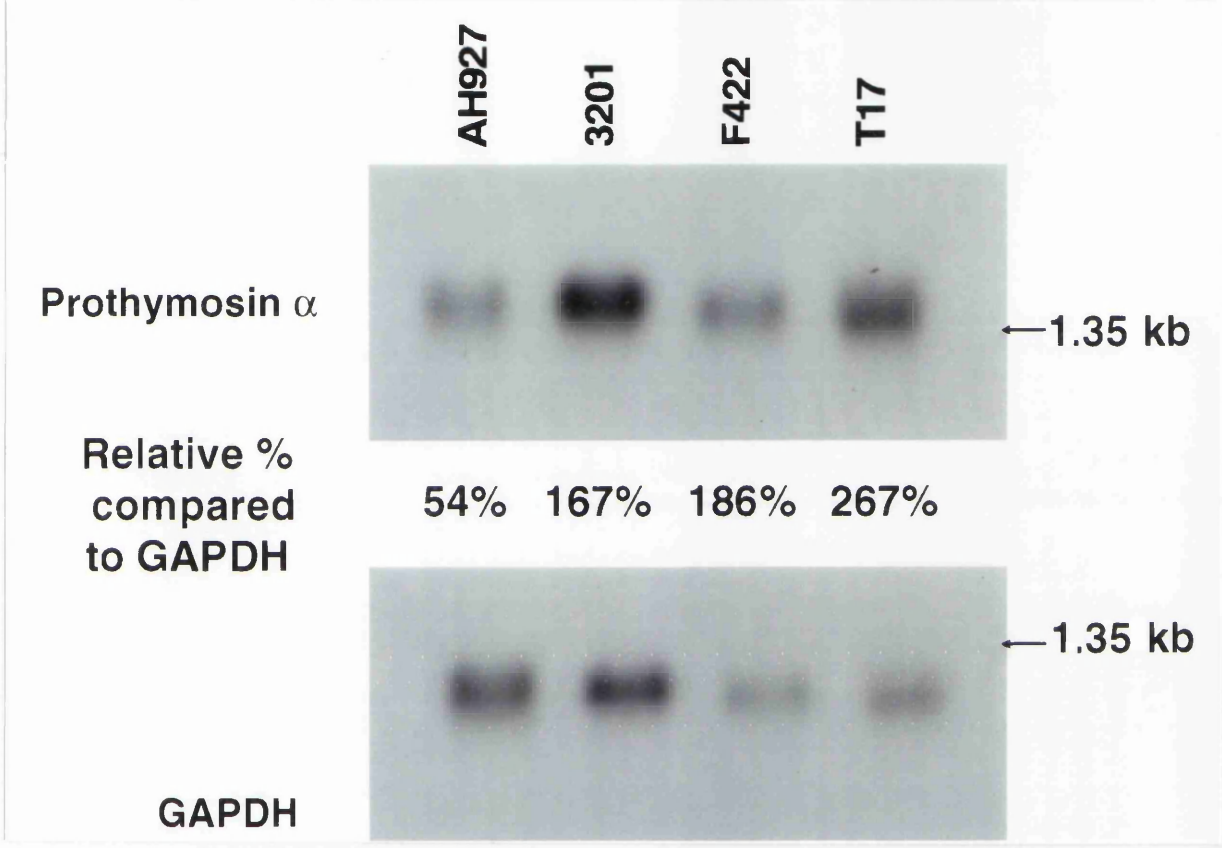


Figure 8.2 Prothymosin-α transcription in lymphoma cell lines.

The prothymosin transcript is ~1.4 kb, the closest size markers are shown.

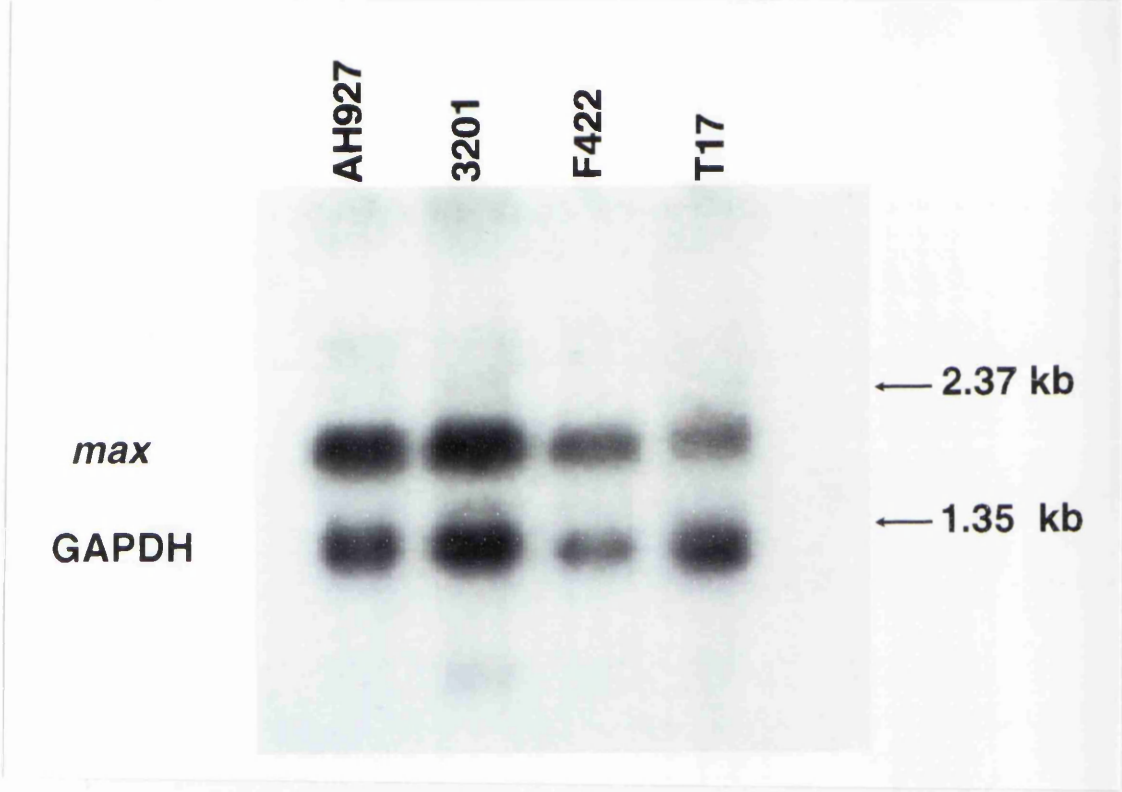


Figure 8.4 Transcription of *max* in lymphoma cell lines.

max transcripts shown at ~2.3 kb.

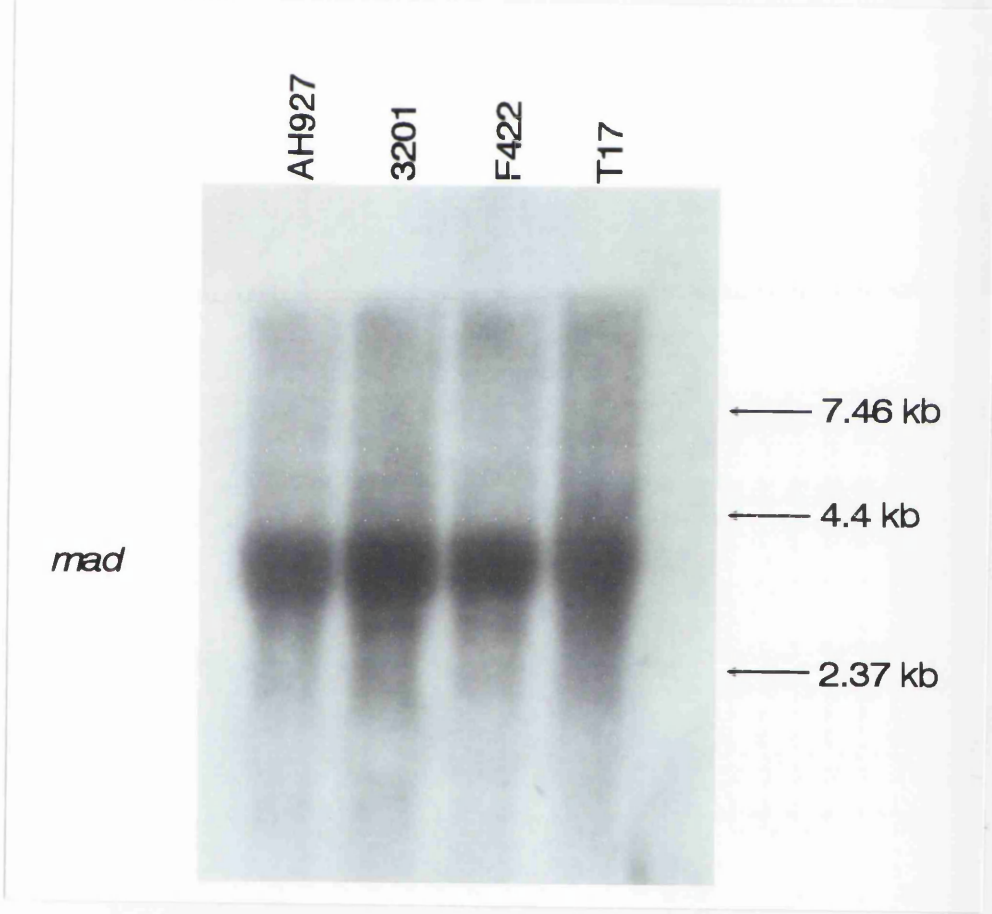


Figure 8.5 Transcription of *mad* in lymphoma cell lines.

mad transcripts are shown at ~3.5 kb.

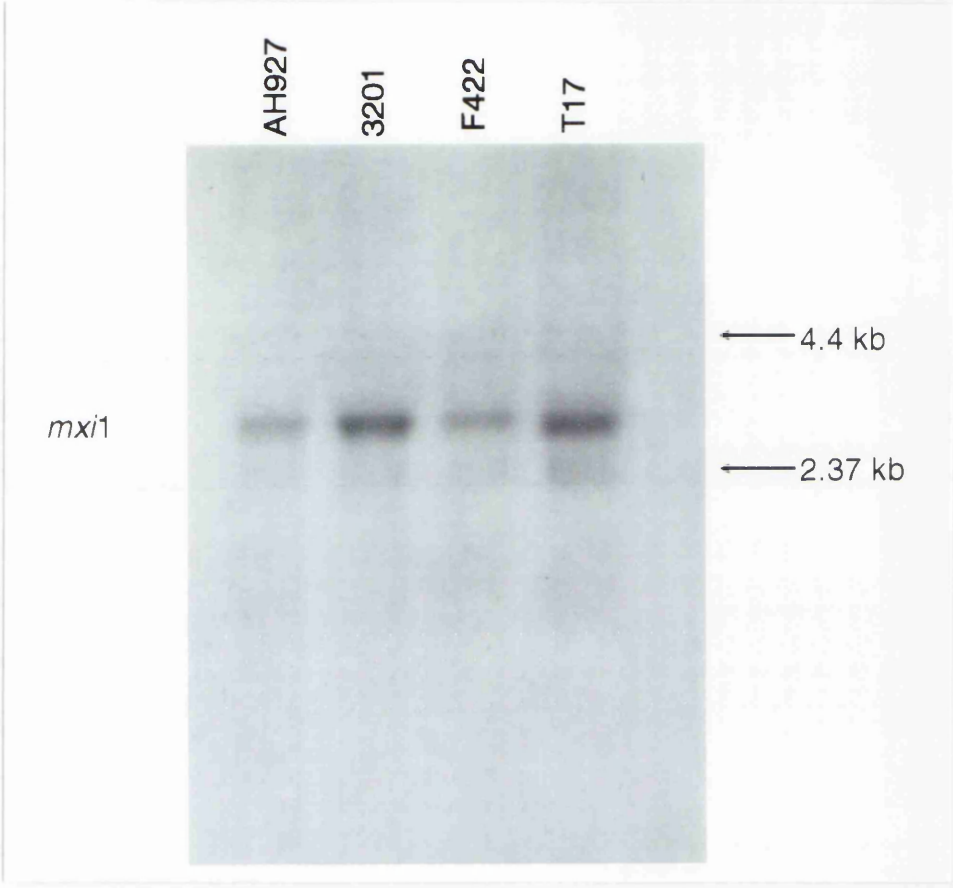


Figure 8.6 Transcription of *mxi*1 in lymphoma cell lines.

*mxi*1 transcripts shown at ~2.8 kb.

8.4 Discussion

Although c-Myc has many of the characteristics of a transcription factor, including its BR-HLH-LZ domain, its acidic and its glutamine rich domains, the number of genes shown to be directly affected by Myc remains small. This is despite the large number of studies on Myc as revealed in the publication of 3300 papers which contain the term Myc in their title or abstract, during the period 1990-1994 (251). This provided the rationale for my analysis of ornithine decarboxylase and prothymosin-α gene expression in the feline leukaemic cell lines overexpressing Myc, as ODC and prothymosin-α, which as previously discussed, are two of the most likely candidates for direct Myc regulation. In this study no deficit in transcription of these genes was found in the T17 cell line. This is despite the lowered transcriptional activity demonstrated by the T17-Myc N-terminal in the yeast assay when linked to the LexA DNA binding domain (chapter 7), and the lack of a discernible phenotype in the chick embryo fibroblast transformation assay chimaera when linked to the normal c-Myc C-terminus (chapter 5). However, this result does not exclude the possibility that T17-Myc is able to interact via its N-terminal domain with either T-cell specific transcription factors, or other developmental stage-specific transcription factors not present in the assays used. Indeed the fact that the T17 cell line shows greater levels of prothymosin-α transcripts than any of the other cell lines, argues that cell type specific factors may be important.

Another obvious explanation is that the T17-Myc protein is an inactive protein, and that the ODC and prothymosin-α genes are transcriptionally regulated by factors other than Myc and Max. However, one problem with this argument is that there is no apparent reason why deregulation of c-Myc should lead to a rise in the transcription of these two genes. Although it could be argued that the effects are not related and one is not a direct result of the other, the evidence for a direct interaction by Myc and Max in regulating these genes is

relatively good. For example, disruption of the Myc/Max binding site on reporter constructs of these two genes prevents the activation of the reporter genes (241,244). One other possibility is that the role Myc plays in the transcription of these genes is somewhat redundant, and transcription can continue in the absence of Myc.

Another intriguing aspect of ODC is that this enzyme appears to be involved in Myc induced apoptosis (252). Using interleukin-3 (IL-3) dependent murine myeloid 32D.3 cells, and overexpressing c-Myc, it was shown that levels of ODC could be increased, but that these cells then died by apoptosis on withdrawal of IL-3. It was also shown that overexpression of ODC and subsequent withdrawal of IL-3 led to the same phenomenon, in a manner dependent on the level of ODC, but this could be blocked by the irreversible ODC enzyme inhibitor α-difluoromethylornithine (DEMO). Further to these findings it was found that rates of death in c-Myc 32D.3 clones were reduced on withdrawal of IL-3 but only in the presence of DEMO. These findings suggest a link between a gene putatively under the direct transcriptional control of Myc, and the apparent ability of Myc to induce apoptosis under some circumstances (63,88,89).

Lately, further evidence has been reported as to how Myc/Max regulation of prothymosin-α and ODC takes place (253). This work has tracked the Myc/Max binding site for the prothymosin-α and ODC genes to the first intron for both genes. However, according to the results of this group, transcriptional activation of Myc/Max is under the negative control of the AP-2 transcription factor (254,255), the binding site for which was found to be contained in regions of extended sequence homology not accounted for by the known Myc/Max E-box motif, within the first intron of both genes (253).

The AP-2 binding sites were found to overlap the Myc/Max binding sites, as shown by DNase I footprint analysis. Negative regulation of Myc/Max was detected by two methods.

Firstly, the AP-2 protein was able to compete more efficiently for binding sites than Myc/Max, and so displace pre-bound Myc/Max in an EMSA. Also, transient transfection assays with reporter constructs activated using the putative Myc/Max-AP-1 binding sites to transactivate the reporter, showed suppression of the reporter in a dose-dependent manner when a CMV-AP-2 expression plasmid was cotransfected. Secondly, it could be demonstrated that the C-terminus of AP-2 interacted with the BR-HLH-LZ of Myc without preventing Max association, but inhibited Myc/Max binding to DNA (253).

The ability of the BR-HLH-LZ proteins Max, Mad and Mxi1, to control Myc function is a topic of current interest. Overexpression of Max has been demonstrated to reduce the incidence of Myc induced tumours in transgenic mice (256). However, this inhibition of Myc function might have no real physiological relevance, as no reports exist of Max being highly expressed naturally in a manner suggestive of a role preventing tumour development. Although if this mechanism was feasible in a natural context one would presume that we would not necessarily know about it, as tumours would not arise, and the mechanism could only be discovered by looking at the gene regulation in healthy animals. Further, there have been no reports in the literature where disruption of *max* has been detected in tumours. Indeed work by myself and Dr. C. Tsatsanis using the feline *max* gene to screen a large number of feline tumour DNAs could not detect any changes in the germ line configuration of the tumours tested (not shown). Another more intriguing feature of Max, is the 9 codon splice variant which seems to be invariant across species (115,218,220). The extra 9 amino acids coded for by the Max 9 protein would logically have some function due to the invariance across species. It is certainly plausible that this variant allows interactions to take place between other members of the transcriptional machinery not available to the other splice variant, in much the same way as residues 105-114 of SV40 large T antigen allow interaction with p107 (257,258). Other *max* mRNAs have been reported to exist, which would result in truncated forms of Max protein if translated (249,259,260).

However, only one, dMax, has been demonstrated to exist in the protein form *in vivo*, and has been found complexed with Myc in immunoprecipitates from cell lysates (261). This deleted form lacks the basic region, helix 1 and the loop, and although able to complex with Myc the resulting Myc/dMax complexes did not bind to oligonucleotides, containing the requisite E-box, in an EMSA. Further, dMax has been shown to repress transcription from a CAT reporter construct in a dose-dependent manner (261).

Further evidence that Myc/Max play a role in transcription of ODC comes from the demonstration that overexpression of Mxi1 can downmodulate an ODC reporter construct, and also decreased endogenous ODC expression by up to 90% in proliferating cells (262). However, suppression of reporter constructs and endogenous ODC never reached 100%, leading to the suggestion that Myc/Max upregulate transcription of this gene, but are not required for basal transcription.

Evidence has come to light recently that there is a second splice variant of Mxi1, which encodes a short amino terminal alpha helical domain (263). This stretch of amino acids is responsible for suppressing Myc activity by recruiting a putative transcriptional repressor which bears structural homology to the yeast transcriptional repressor SIN3 (132). Mad has also been demonstrated to bind to the SIN3 repressor. Furthermore it has been demonstrated that there are two main forms of the mammalian homologue, mSin3A and mSin3B, with a further 9 amino acid splice variant of the mSin3A gene (mSin3A9) (133). It is postulated that the mammalian Sin3 proteins act as transcriptional repressors by tethering Mad-Max complexes to DNA in a ternary complex (132,133).

In conclusion, the popular idea that Myc regulated transcription is repressed by an excess Mad/Max or Mxi1/Max appears too simplistic. This initial hypothesis stemmed from the finding that *mxi*1 and *mad* mRNA and protein levels increased as those of *myc* decreased in

various cell types during terminal differentiation (122, 123, 250, 284). It now appears that different forms of Mxi1, termed strong repressor and weak repressor, serve different functions, with the weakly repressing form attenuating the strong repressor by competing for target sequences and / or accessory proteins via the shared carboxy-terminal (132), while the same may, or may not, hold true for Mad.

CHAPTER 9

Chapter 9 General Discussion

9.1 Evolution of the T17 *myc* sequence

The work carried out for this thesis sheds further light on the complex picture of the mechanism of action of c-*myc*, since the T17-*myc* mutant retains oncogenic potential *in vivo*, but seems incapable of reproducing many of the parameters associated with oncogenes when assayed *in vitro*. These observations contradict the previously held assumption that the coding sequences of the second and third exons are always intact in tumours (33). Moreover, the impaired ability of the mutant to activate transcription does not appear to be a bar to oncogenic potential, but is consistent with several recent studies which suggest that N-terminal coding sequence mutations of c-*myc* are common in tumours (231,232,234). Figure 9.1 demonstrates properties of Myc proteins. The functional domain TA1 (138) is equivalent to the region maintained in T17-Myc, and is likely to be responsible for the transcriptional activation measured in the yeast assay (Chapter 7).

One of the most intriguing aspects of the T17 *myc* gene is how such a complex set of mutations evolved. The question arises whether the deletions at the N-terminus or the insertion at the C-terminus occurred first. Indeed it is possible that the bipartite deletion was a temporally two-step process, rather than both deletions occurring in the transduced gene during a single replication cycle. Another possibility, is that the bipartite deletion arose as a large single deletion, with a subsequent recombination event leading to the sequence regaining the 8 bases separating the deletions. The problem posed is whether the N-, or the C-terminal mutations have independent effects on the transformation of lymphocytes, or whether both mutated domains were necessary for the genesis of tumours. As outlined above, mutations in the N-terminal domain seem to be prevalent in lymphomas, suggesting that the N-terminal mutations may be the first, and most important. In this

model the first step would be loss of transcriptional activity from the N-terminus, leading to, e.g. a growth advantage, such as viral propagation in non-lymphoid cells where the transduced gene would not induce apoptosis. Alternatively prevention of differentiation of cells containing the transduced gene is another possibility. In either case the C-terminal mutation would evolve later as an adjunct. As the C-terminal mutation removes the increased growth rate and inhibits growth in soft agar in CEF cells (Chapter 5), it seems likely that this mutation may augment the loss of transcriptional activity caused by the N-terminal mutations.

However, the fact that mutations in the N-terminal of Myc occur at high frequency in lymphomas does not discount the possibility that the C-terminal mutation arose first. An alternative model then presents itself, where titration of Max without transcription is the important first step to transformation of lymphocytes. In this scenario the evolution of the N-terminal mutations could help in transformation by making the protein product of the transduced gene more stable, thereby making more mutant Myc available for complexing with Max.

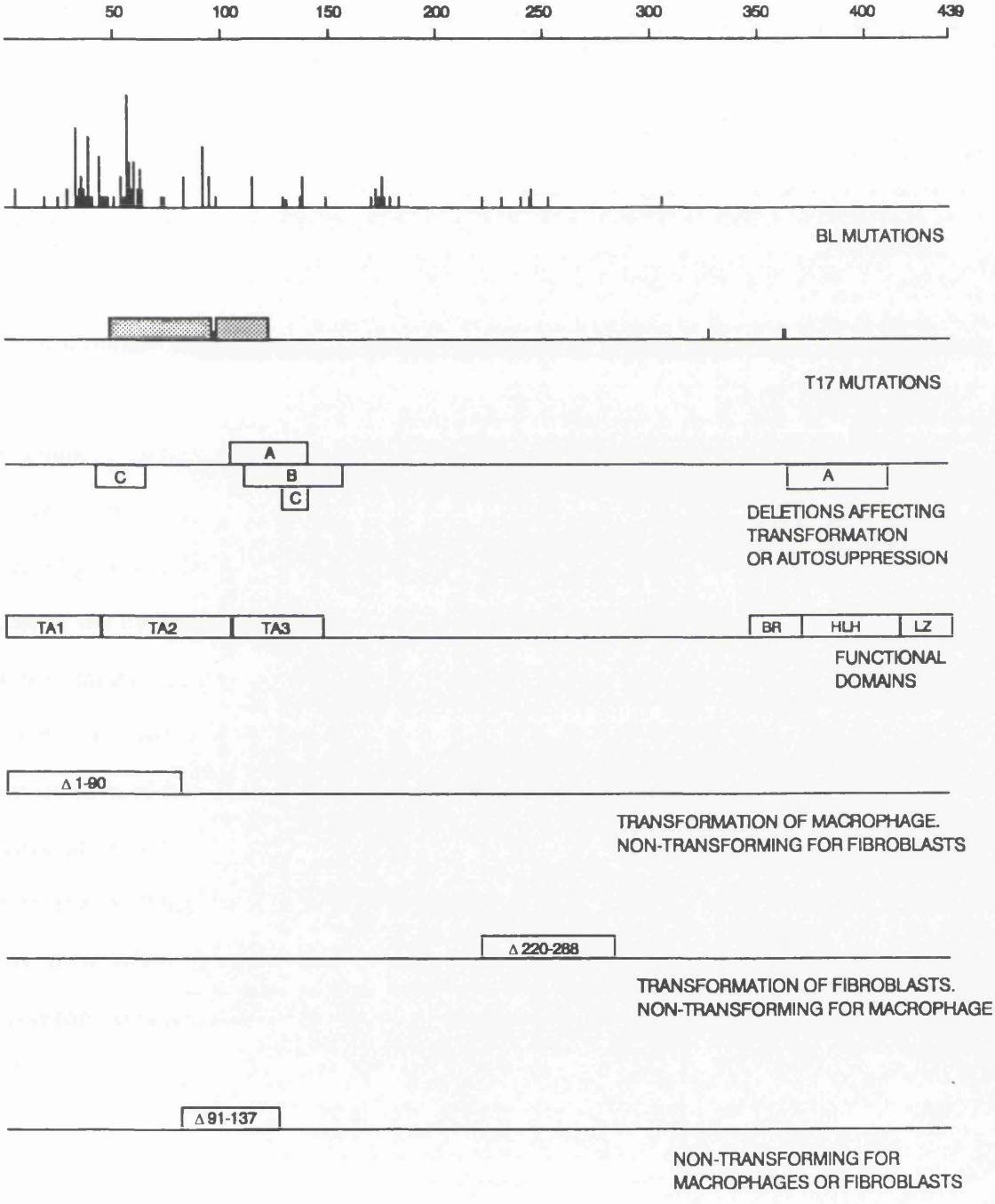


Figure 9.1 Properties of Myc proteins. Adapted from (189) and table 4.1.

Figure 9.1 Properties of Myc proteins.

Legend: this figure shows the location of mutations in T17-Myc (stippled boxes are deletions), with vertical lines denoting point mutations found in Burkitt's lymphomas (230-232), the height of each line is proportional to the number of mutations found at that location. Deletions affecting transformation or autosuppression are taken from references (203, 208) [A], (212) [B] and (281) [C]. Deletions affecting different cell types are shown in boxes with Δ followed by the amino acids deleted (from table 4.1 references (202, 212)).

9.2 C-terminal mutation

Perhaps one of the most puzzling features of the T17-Myc mutant is that the basic region L>FR mutation does not prevent binding to the consensus 5'-CACGTG-3' motif (chapter 6). By using computer models which depict the putative helical wheel structure (GCG, Wisconsin, 'Helical Wheel'), the basic region is predicted to be disrupted (figure 9.2). However, the histidine residue which is proposed to make direct contact with the thymine methyl group at the heart of the 5'-CACGTG-3' motif (264) is in the same relative position. Loss of the hydrophobic leucine may be tolerated by replacement with a hydrophobic phenylalanine at the same point in the helical wheel. Thus it may well be that the disruption of the rest of the helical wheel structure does not significantly affect the ability of the protein to bind its DNA motif. This hypothesis however, does not account for the dissociation of transformation and growth rate parameters by the BR mutation as described in chapter 5. This is unlikely to be a property specific to the feline Myc, as the same result was found when this mutation was introduced into the chicken c-*myc* gene (265). Therefore, it is possible that the basic region mutation is able to either target a specific subset of Myc regulated genes, or prevent targeting of certain gene(s).

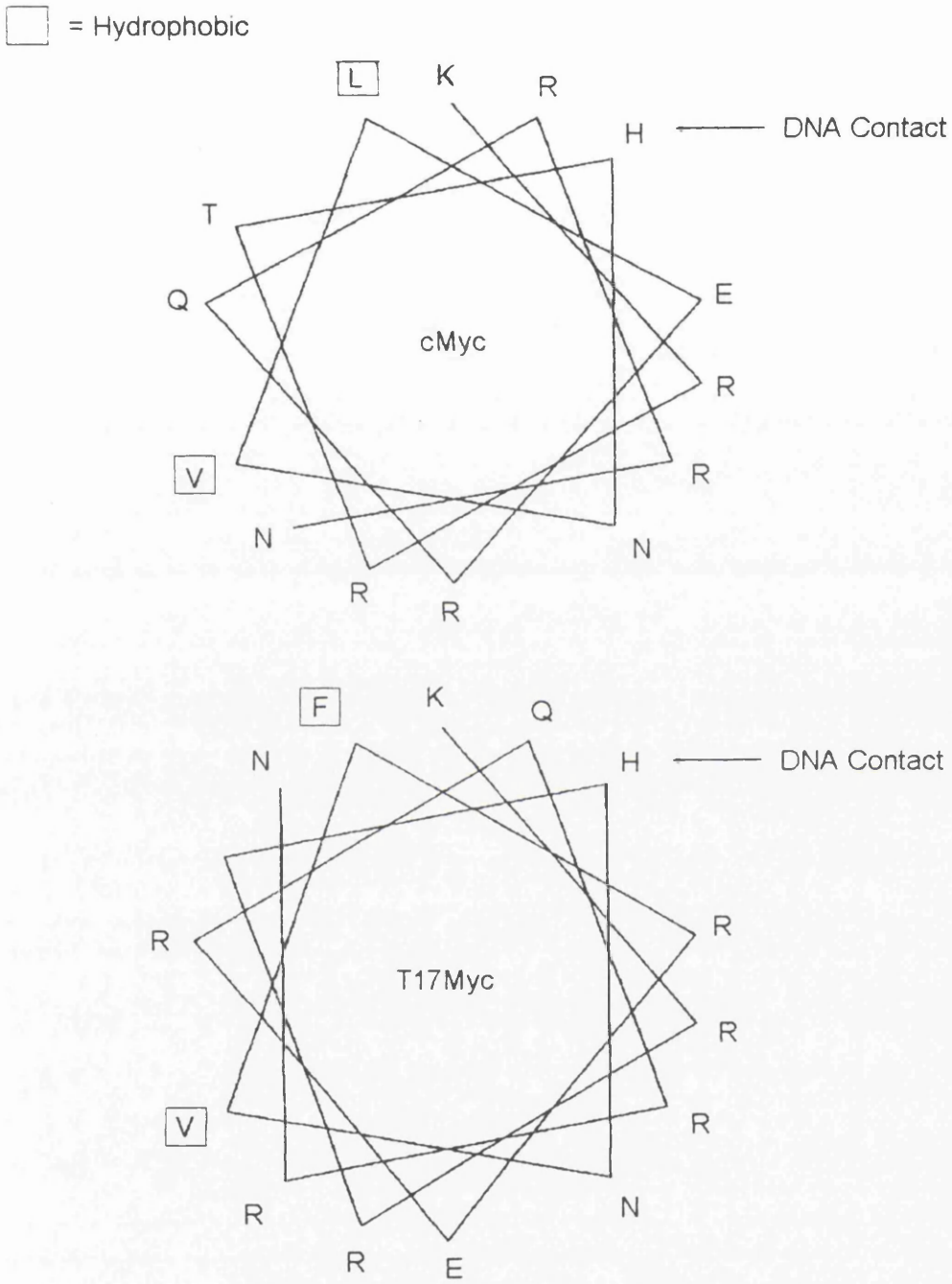


Figure 9.2 Helical wheel structure of c-, and v-Myc basic regions.

Another possibility is that site specific binding to DNA is necessary but not sufficient to trigger transcription. It has been demonstrated that proteins binding to DNA can induce a bend in the DNA by asymmetric phosphate neutralisation (266). Indeed binding of Jun:Jun homodimers bends DNA towards the minor groove, whereas Fos:Jun heterodimers, binding to the same site, bend DNA towards the major groove (267). Thus control of transcription may well depend to some extent on the direction and/or magnitude of bending of DNA. Regulation of transcription might be brought about by one of a number of mechanisms, such as; bringing distantly bound transcription factors close together to facilitate DNA-looping; mediation of interactions between transcription factors and the general transcription machinery, where the DNA is wrapped around the protein complex; energy stored in a protein-induced bend used to favour formation of an open transcription complex or dissociate RNA polymerase in the transition from initiation to elongation (268). While the T17-Myc protein retains the ability to bind DNA it may have altered potential to induce DNA bending due to the basic region mutation, and this could account for the lack of growth rate acceleration observed in the c/v-Myc chimaera in chapter 5.

Such an hypothesis would also take into account the change of leucine to phenyalanine, if the hydrophobic leucine is shown to intercalate into the DNA. It is becoming clear that intercalation of DNA by hydrophobic residues of transcription factors can lead to 'kinking' in the DNA (269). Two types of intercalation have been recognised: partial and complete. Partial intercalation requires that the intruding side chain unstacks two adjacent base pairs, but does not itself stack in the space left in the helix. Complete side chain intercalation leads to the hydrophobic side chain being stacked over a base pair in its entirety (269). Side chain intercalating proteins do not display any particular structural motif, with regards to their DNA binding surface and no simple method exists of predicting if a particular residue will intercalate.

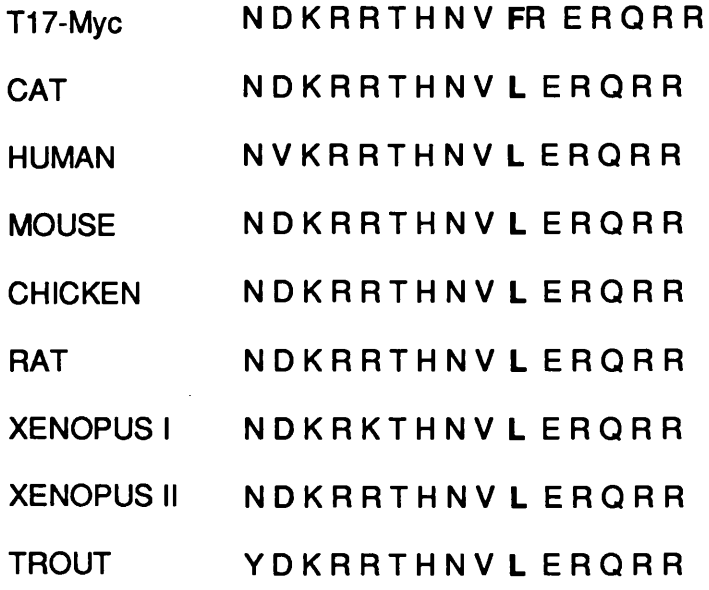


Figure showing the basic region of c-Myc proteins from different species.
The invariant hydrophobic leucine is shown in bold type, with the phenylalanine residue in the equivalent position of the T17-Myc protein also shown in bold.

Figure 9.3

However, the fact that Myc family proteins maintain the leucine residue across species (270) (Figure 9.3), argues strongly that this residue has an important function which could well be DNA intercalation. Thus, we might expect that the phenylalanine residue of the T17-Myc basic region will induce a 'kink' different from that induced by the c-Myc leucine. This differential DNA bending hypothesis should be testable using the techniques set out in Strauss and Maher (266).

9.3 N-terminal mutations

Early work on transcriptional regulation by c-Myc suggested that transcription could be either activated or repressed by c-Myc (271). Furthermore the ability to repress transcription from the mouse metallothionein promoter was lost when 138 amino acids (between 40 and 178) were deleted from exon 2 of c-Myc, although activation from the heat shock protein 70 promoter was maintained (271). Thus transcriptional repression by c-Myc might be the mechanism by which the T17-Myc mutant exerts its oncogenic effect.

More recent evidence has suggested that repression of thrombospondin 1 might be mediated by c-Myc (272). Thrombospondin 1 is a secreted glycoprotein known to inhibit tumour neovascularization, therefore its repression by Myc is suggested to be a further link between Myc and neoplasia (272).

The fact remains, however, that despite a very large research effort there is little evidence that c-Myc is directly responsible for the transcription or repression of significant number of genes. Certainly there is little evidence to support the notion that those genes mooted as direct transcriptional targets are responsible for the pleiotropic effects ascribed to c-Myc, i.e. cell cycle control, differentiation, oncogenicity and apoptosis. Therefore it may be that

our concepts of c-Myc function are wrong. It may be that c-Myc does not act as a transcription factor in the conventional sense, whereby a specific gene is activated or repressed, rather it may have some, as yet, undefined function. What function might c-Myc possess that could account for the observed effects? One possibility is that the Myc/Max dimer acts by tethering components of the transcriptional machinery to target genes allowing transcription factor complexes to accumulate. This mechanism may be like an enhancer function which is not required for basal transcription of target genes, but allows rapid upregulation/downregulation of a variety of genes when c-Myc is activated. This might account for the lack of obvious target genes directly transcribed by c-Myc, which seems strange for a protein which has such an apparently central role in the viability of an individual cell, and consequently the whole organism.

One of the least prosaic descriptions of c-Myc was as a "citadel of incomprehensibility" (129). In many respects this description still applies, for although a great deal of knowledge has accrued on Myc interactions with other proteins in binding DNA, and how transcription from Myc reporter constructs can be suppressed by Mad family, Mxi1, and SIN3 proteins, the mechanism by which Myc actually exerts its oncogenic effects is still unknown. The evidence is exceedingly strong that Myc is a transcription factor, but exactly which genes are positively and/or negatively regulated remains uncertain. Even a gene such as prothymosin-α which has strong supporting experimental evidence for direct transcriptional control by Myc/Max (166), is not universally agreed to be a target for Myc regulation, and the assertion that Myc contributes to prothymosin transcription has been challenged (273). Hence the difficulty in determining the role which the T17Myc protein plays in oncogenesis.

9.4 The problem of defining the role of c-Myc

The problem of defining the actual function of c-Myc is unresolved. To this end I would like to suggest an alternative strategy for investigation of c-Myc function using yeast

artificial chromosomes (YAC). Yeast artificial chromosomes were originally developed for research into the structure and behaviour of eukaryotic chromosomes, e.g. during meiosis, and for use in cloning very large genes including introns (274). A complete human DNA library can be constructed with 60,000 clones (assuming 150 kb fragments), as opposed to $>2.5x10^5$ clones in the bacterial vector with the greatest capacity, the cosmid.

I suggest that the YAC system could be used to assist in identifying genes which are transcriptionally regulated by Myc/Max. The problem is one of conflicting results from experiments on Myc-regulated genes, e.g. prothymosin-α and cyclin D1 where one group finds regulation (166,172) but this is refuted, or the opposite kind of regulation is found by other workers (48,273). The problem may be that reporter plasmids do not adequately reproduce the complexity of chromatin in eukaryotic cells. The main structural problem which eukaryotic transcription factors have to contend with is the nucleosome. This structure consists of an octamer of two of each of the nucleosomal histones; H2A, H2B, H3, and H4. H3 and H4 form the inner core and are among the most highly conserved of all proteins. 146bp of DNA is wrapped 1.8 times round the octamer, and a further histone, H1, binds across the entry and exit points of the DNA from the core (275). Nucleosomes are dynamic structures which appear to be removed from promoter and enhancer elements before, or concurrent with *in vivo* gene activation (276). Replication-independent nucleosome disruption has been found in the yeast *PHO5* promoter, where the PHO4 activator disrupts four nucleosomes covering the *PHO5* promoter (277). PHO4 recognises the same core 5'-CACGTG-3' DNA consensus motif that is bound by Myc/Max (120). While this is not proof that c-Myc has the same property, it does suggest the possibility.

The complexity of a eukaryotic chromosome is not readily matched by a transfected reporter construct, especially if it remains episomal. Even a stably integrated reporter gene construct may give anomalous results. To select viable clones, the integration process must

give a fully functional selectable marker gene, such as G418 resistance, or the cells die. However, there is no selection pressure to keep the reporter gene, or its promoter region, intact. This leads to the problem that the reporter gene assay may give a definite positive result, but a negative result is not definitive. Other than nucleosome displacement, the possible importance of DNA bending in transcriptional control (268) is another aspect unlikely to play the same role in transcription from reporter constructs. Thus my proposal is twofold. Firstly, large pieces of DNA containing putative targets of Myc/Max could be cloned in their entirety into YACs and transcription could be measured from induced Myc/Max proteins (141), without interference from endogenous Myc or Max. In this system northern blot analysis, or RNase protection, of the induced cells would be undertaken to look for mRNA transcripts from the gene of interest, rather than induction of a reporter such as β-galactosidase. As the chromosomal locations of some proposed target genes are known it may be possible to identify and obtain YACs for this purpose.

The second part of the proposal is, however, more difficult. This would consist of using a human genomic DNA library cloned into YACs, which would be introduced into yeast containing inducible c-*myc* and *max* constructs. Subtractive hybridisation of uninduced from induced cells, could then be used to search for genes transactivated by Myc/Max. Such an approach, although difficult, could prove very fruitful as genes which may be basaly transcribed in mammalian cells, but upregulated by Myc/Max, would hopefully not be transcribed in yeast. Despite the obvious drawback that genes downregulated by Myc would not be detected, this procedure could at least be used to screen for positively regulated genes.

Either of the two proposed methods would, in principle, provide a novel test of Myc/Max mediated transcription. Although yeast chromatin is not exactly equivalent to its

mammalian counterpart, e.g. yeast does not appear to have histone H1 (150), it could provide a more sensitive method to examine Myc/Max transcription than reporter plasmids.

As outlined in chapter 7, loss of transcriptional activation may be the means by which constitutively activated Myc in Burkitt's lymphomas allows escape from cell cycle control, with mutations occurring frequently in the Myc N-terminal domain of these cancers (230,232-234). This region has also been demonstrated to be glycosylated with O-linked *N*-acetylglucosamine (278). The significance of glycosylation has not been determined, but it appears to have some relationship to protein phosphorylation, and other transcription factors, such as Fos and Jun, also carry O-GlcNAc moieties (278). Figure 9.1 outlines the T17-Myc mutations as well as regions shown to be important for transformation of different cell types, and proposed transcriptional activation domains.

9.5 Summary

In summary, the T17-Myc mutant is far from completely understood. Several lines of investigation are open to further study. These include defining its *in vivo* oncogenic properties more precisely, in a more easily controlled system such as a mouse model. To this end T17-*myc* and feline c-*myc* have been cloned into murine retroviral vectors, and *in vivo* experiments will yield further information on the transforming potential of the mutant. Another avenue of research would be the use the avian retroviral constructs described in this thesis to ascertain the abilty of the T17, and chimaeric Myc constructs to transform avian bone marrow cells (202,212,216). Further experiments should also be carried out to investigate the unique stability of the T17-Myc protein, while *in vitro* DNA binding assays may determine whether the T17-Myc basic region has an altered ability to bend or kink DNA. Finally, it may also prove fruitful to construct retroviral vectors containing separated

N-terminal deletions to ascertain whether both parts of the deletion are required for oncogenicity.

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