A STUDY OF THE *myc* GENE IN FELINE LEUKAEMIAS

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ABBREVIATIONS

ALV	avian leukosis virus
ATP	adenosine triphosphate
BLV	bovine leukaemia virus
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleoside triphosphate
dTTP	deoxythymidine triphophate
DEP	diethyl pyrocarbonate
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
FeLV	feline leukaemia virus
HTLV	human T-lymphotropic virus
IL2	interleukin 2
IL3	interleukin 3
kb	kilobases
LTR	long terminal repeat
MOPS	sodium morpholinopropane sulphonic acid
MMTV	mouse mammary tumour virus
MuLV	murine leukaemia virus
PDGF	platelet derived growth factor
PIPES	piperazine-N,N'-bis[2-ethane sulphonic acid]
SDS	sodium dodecyl sulphate
SFFV	spleen focus-forming virus
TCR	T-cell antigen receptor
TEMED	N,N,N',N'-tetramethylethylenediamine
X-gal	5-bromo-4-chloro-3-indolyl-β-galactoside

LIST OF CONTENTS

LISTS OF TABLES AND FIGURES

SUMMARY

I isolated and characterised a clone of the normal feline c-myc gene. Sequence analysis showed the gene to be highly related in other mammals but less well related in the chicken. The feline c-myc gene possessed an apparently non-coding first exon with a dual promoter structure, similar to that found in the human and mouse c-myc genes. The sequences of three independent FeLV v-myc genes were compared to that of the c-myc gene to identify possible structural alterations involved in myc oncogenic activation. The c-myc clone also provided probes to map c-myc rearrangements in feline thymic lymphosarcomas. Some rearrangements were due to FeLV integration within or upstream of c-myc, but one case involved a complex 3' alteration which was apparently not directly virus-induced.

S1 nuclease mapping of RNA from normal cells using c-myc probes located 5' discontinuities to each of the two promoter-like sequences (P1 and P2), and a major 3' discontinuity mapping to the most 3' of two possible polyadenylation signals. Tumours carrying c-myc rearrangements did not display readily obvious abnormalities in the structure or levels of c-myc RNA, except for case T24 which appeared to contain RNA lacking exon 1 sequences. However, the ratio of P1 to P2 RNAs detected in tumours varied considerably and was high in tumours with a rearrangement adjacent to c-myc, although it was equally high in some tumours with an ostensibly normal c-myc gene structure. There was a consistent lack of detectable RNA from normal c-myc alleles in tumours containing different myc-transducing FeLVs. Also, in tumour T24 which expressed a rearranged c-myc gene, RNA from the normal c-myc allele could not be detected.

The phenotype of thymic tumours was characterised with respect to expression of RNA of the α and β-chains of the T-cell antigen receptor (TCR). Several tumours, including all those induced by two myc-transducing FeLVs and others carrying a rearranged c-myc gene, contained TCR α and β-chain transcripts. This study provided preliminary evidence that one tumour contained independently transduced myc and β-chain TCR genes present in separate FeLV proviruses, suggesting a direct role for TCR genes in oncogenesis.

CHAPTER ONE

GENERAL INTRODUCTION

Feline leukaemia virus (FeLV) is a retrovirus and is an important pathogen of the domestic cat. Infection can result in a variety of diseases including degenerative and proliferative conditions (Jarrett, 1984). This thesis concerns the molecular mechanisms by which FeLV induces leukaemias, especially thymic lymphosarcomas, the most common neoplasm associated with the virus. The introduction first considers some mechanisms of oncogenesis by retroviruses, then outlines features of FeLV pathogenesis. Finally, I discuss the myc gene since much of my work concerns the involvement of this gene in feline leukaemias.

1.1 THEORIES OF ONCOGENESIS

1.1.1 General

There have been various theories concerning mechanisms of oncogenesis. The somatic mutation hypothesis of Boveri in 1914 proposed that normal cells became transformed due to genetic changes associated with chromosomal abnormalities (Ruddon, 1981). This idea has gained support through cytogenetic analysis showing that many tumours display characteristic chromosomal rearrangements (Rowley, 1984). Further support for a genetic basis for cancer has been provided by study of tumours induced by chemical carcinogens which have been associated with mutations in specific cellular genes (Barbacid, 1986). Alternative evidence linking genetic changes with neoplasia was provided by observations that susceptibility to some human tumours, such as retinoblastoma, could be associated with inherited genetic defects (Knudsen, 1971).

Different views have held that oncogenic mutations may activate a dominant transforming gene or alternatively inactivate some suppressor function to release cells from normal growth restraints (Murphree & Benedict, 1984). However, mutational theories of cancer do not have unanimous support and another perspective has been that epigenetic factors rather than mutations contribute to cancer development (Rubin, 1980).

1.1.2 Retroviruses and oncogenesis

Viruses have been implicated as causal agents in tumours since

Ellerman and Bang in 1908 and Rous in 1911 showed that cell-free extracts from chicken leukaemias and sarcomas could transmit these diseases. These and similar studies with mouse mammary tumours and leukaemias led to the identification of retroviruses as oncogenic agents (Weiss et al, 1985). In 1964, transmission of feline lymphosarcoma with cell-free extracts led to the discovery of feline leukaemia virus (Jarrett et al, 1964).

Detailed molecular theories of retrovirus-induced oncogenesis have developed only relatively recently as the integrative replication-cycle of these RNA tumour viruses has been understood. Early ideas focused on genetic mechanisms whereby expression of viral genes was necessary for transformation. An aspect of the "protovirus hypothesis" of Temin (1976) was that an acutely oncogenic virus incorporated a cell-derived cancer gene by recombination. An alternative view was the "oncogene hypothesis" of Huebner & Todaro (1969). They proposed that many vertebrates contained endogenous viruses or "virogenes" which included an "oncogene" sequence. Normally these genes would be suppressed but carcinogenic agents might derepress their expression. Some elements of both hypotheses proved correct as a wealth of recent evidence showed that some, but by no means all, oncogenic retroviruses carry specific transforming genes, or oncogenes (section 1.2).

1.1.3 Multistep oncogenesis

Recognition that retroviral oncogenes, or v-onc genes, were modified versions of normal cellular genes (proto-oncogenes) (Bishop, 1983) provided a link between viral and non-viral theories of oncogenesis, since it was apparent that the transforming potential of a proto-oncogene might be activated by different agents including viruses, chemical carcinogens or chromosomal abnormalities. Tumour development has been viewed as a multistep process based on genetic and epidemiological observations and experimental studies with chemical carcinogens (Ashley, 1969; Knudsen, 1971). The discovery and functional study of oncogenes suggested possible molecular events underlying these stepwise phenotypic changes (Klein & Klein, 1985). However, evidence for epigenetic mechanisms in oncogenesis suggested that the contributory events need not all be mutational changes, and the cellular environment and host's physiological status may also play a part (Rubin, 1980; Barbacid, 1986).

1.2 RETROVIRAL ONCOGENES

1.2.1 The src gene

The first v-onc gene to be discovered was the src gene of Rous sarcoma virus (RSV) which illustrates some general features of other v-onc genes. Study of RSV mutants by Toyoshima & Vogt (1969) and Martin (1970) showed the RSV transforming gene to be distinct from the viral replicative genes and that its continuous expression was required to maintain the transformed state. After physical characterisation of the gene, called src, use of a src-specific hybridisation probe yielded the remarkable finding that normal, uninfected chicken DNA contained a src-related gene (Stehelin et al, 1976). Other vertebrates were also found to contain a src-related gene.

More than twenty v-onc genes have now been identified in avian, murine, feline and simian retroviruses and a general property seems to be that related genes (c-onc genes) are present in normal DNA from diverse species (Bishop, 1985). This finding had important implications as discussed below. However, it should be mentioned that not all retroviral oncogenes need fit this category. One example seems to be that induction of murine erythroleukaemia by SFFV requires expression of an unusual viral recombinant env product rather than a cell-derived sequence (Linemeyer et al, 1982).

1.2.2 The origin of viral oncogenes

The relationship of v-onc genes to normal cellular genes suggested that a v-onc gene was a captured version of a c-onc gene as predicted by Temin's protovirus hypothesis. Models for the capture of cellular sequences by retroviruses have been based on structural comparison of v-onc and proto-oncogene sequences (Bishop, 1985). The structure of most viral oncogenes is consistent with the model of Swanstrom et al (1983) for transduction of the src gene by RSV. This proposes that initial recombination occurs at the DNA level as a result of proviral integration. The provirus integrates 5' to the gene to be transduced, then a deletion leads to generation of viral-cellular gene fusion transcripts, which may be spliced and packaged into virus particles. A second recombination step between this RNA and an intact viral RNA genome would provide the 3' end of the recombinant viral genome. The absence of introns from v-onc genes and the presence of a poly(A) tail

at the 3' end of a v-fps gene support the involvement of an RNA intermediate (Huang et al, 1985).

This need not be the only possible mechanism, and another model considered the formation of oncogenic retroviruses from cellular movable genetic elements (Temin, 1980). Interestingly, the v-ski gene of SK virus is flanked by small direct repeats similar to those occurring at each end of inserted transposable elements (van Beveren et al, 1985).

1.2.3 The role of cellular oncogenes

The wide occurrence of genes related to v-onc genes in different species agreed in some ways with the oncogene hypothesis of Huebner & Todaro. But contrary to their hypothesis, c-onc genes normally were not associated with viruses and were expressed in normal cells, presumably performing some fundamental function in diverse species.

Some progress has been made in understanding the function of v- and c-onc genes. A major theme links many genes with different stages of cellular growth control networks (Weinberg, 1985). Several src-related products (e.g. abl, erbB, fms) display tyrosine kinase activity and include products related to known growth factor receptors. Their potential role in transmitting external growth signals into the cell is supported by their location at the plasma membrane.

The ras family products may act at a similar level as they are related to G proteins which are involved in control of growth signal transmission from the plasma membrane into the cell (review: Lowy & Willumsen, 1986). In contrast, the sis product may function as a growth factor itself, as it is secreted and is homologous to PDGF (Heldin & Westermark, 1984). A distinct group of oncogenes are those coding for products which are located in the nucleus (myc, myb, fos; Eisenman & Thompson, 1986) leading to speculation that they may control expression of other genes.

1.2.4 Non-viral activation of oncogenes

The presence of proto-oncogenes in normal cells presented the possibility that they may be activated by means other than viral transduction. Direct evidence that tumours may contain active oncogenes which were not associated with viruses was obtained using fibroblast transformation assays with tumour DNA (review: Lowy & Willumsen, 1986). This identified transforming c-ras genes, activated by specific point

mutations. These genes were the cellular homologues of the v-ras genes of murine sarcoma viruses.

Indirect evidence implied that the oncogenic potential of other c-onc genes could be activated by non-viral mechanisms. The c-myc gene, the counterpart of the MC29 virus v-myc gene, was found frequently to be disrupted by characteristic chromosomal translocations in Burkitt's lymphomas (review: Leder et al, 1983). Similarly, the c-abl gene, homologue of the v-abl gene of Abelson-MuLV, was altered on the rearranged Philadelphia chromosome in human myeloid leukaemias (review: Adams, 1985). Another mode of rearrangement of c-onc genes was amplification, as found for example, with the c-myc gene in various human tumours (section 1.6).

1.2.5 Alterations in structure and expression of oncogene products

Transforming genes have been proposed to differ from their normal counterparts by inappropriate expression or by coding changes giving a malfunctioning product. Thus, v-onc genes are highly expressed compared to their normal c-onc progenitors. On the other hand transforming c-ras genes become active through specific point mutations (Lowy & Willumsen, 1986). However, this may be an artificial division, since both factors may be required for oncogenic activation of some genes.

Studies on the v-src gene of one strain of RSV show that coding changes and elevated expression contribute in this case. It was shown that the gene must be expressed at a threshold level to achieve transformation (Jakobovits et al, 1984). However, over-expression of the normal c-src gene did not cause transformation, suggesting that some structural change was also necessary. This v-src gene carried an altered C-terminus coding region and multiple codon changes relative to c-src, but only a single amino acid substitution was required to confer transforming activity if the product was expressed at elevated levels (Levy et al, 1986).

1.3 CIS-ACTIVATION OF CELLULAR ONCOGENES BY PROVIRUSES

1.3.1 General

Many retroviruses which do not appear to carry an oncogene have been classed as weakly oncogenic since in general they do not transform cells in vitro and induce tumours with long latency in vivo. Tumours of chickens, mice and cats induced by these viruses often display a clonal

pattern of integrated proviruses, indicating that they derive from one or a few infected cells (Payne et al, 1981; Fung et al, 1981; Cohen et al, 1979; van der Putten et al, 1979; Casey et al, 1981). This led to proposal of the model of insertional mutagenesis whereby an integrated provirus activates the transforming potential of an adjacent c-onc gene in a cell which then proliferates to form a clonal tumour. This concept was first supported by Hayward, Neel and Astrin (1981) who showed that proviruses were inserted at the c-myc locus in ALV-induced chicken bursal lymphomas.

Many target genes for proviral insertions have now been identified in tumours in different species, by two main approaches. First, some tumours were found to carry insertions at c-onc homologues of known v-onc genes, such as c-erbB in chicken erythroleukaemias, c-myb in mouse lymphoid tumours and c-Ha-ras in a chicken nephroblastoma (Fung et al, 1983; Shen-Ong et al, 1986; Westaway et al, 1986). Also, insertions have been found at loci coding for the known growth factors IL-2 and IL-3 in leukaemic cell lines of the ape and mouse respectively (Chen et al, 1985; Ymer et al, 1985).

Secondly, tumour-specific, common integration sites have been characterised with the expectation that they might represent previously unknown genes with oncogenic potential. This was based on the assumption that proviral integration would not normally occur at specific host sites (Nusse, 1986). Thus, detection of a common integration site in tumours may indicate clonal expansion of a cell which sustained an oncogenic insertion.

This has revealed several novel putative oncogenes. In MuLV-induced thymic lymphomas, the pim-1 and Mlvi loci were frequently found to carry proviral insertions in the mouse and rat respectively (Cuypers et al, 1984; Tsichlis et al, 1983), and in MMTV-induced mammary carcinomas the int-1 and int-2 loci were often disrupted (Nusse & Varmus, 1982; Peters et al, 1983).

Recently, it has been shown that an int-1 gene construct partly transforms epithelial cells, providing experimental evidence that cellular genes identified in this way have transforming function (Brown et al, 1986). Also, the pim-1 sequence indicates that it is related to the protein kinase gene family which includes several known oncogenes (Selten et al, 1986).

1.3.2 Mechanisms of cis-activation

The role of a provirus as an insertional mutagen generally depends on its powerful LTR transcriptional controls which deregulate expression of the adjacent c-onc gene. Additional genetic changes have been reported, such as alteration of the c-onc coding sequence, amplification of the rearranged allele or insertions at other loci. These observations suggested that an oncogenic insertion may initiate cell transformation and other genetic changes may contribute to tumour progression.

Promoter insertion. ALV insertions at the 5' end of c-myc often are in the same transcriptional direction as the gene, and generate ALV-c-myc fusion transcripts initiated in the 3' LTR U5 region (Hayward et al, 1981; Payne et al, 1982). A variation of this theme produces fusion transcripts initiating in the proviral 5' LTR which are spliced onto c-onc gene exons from a viral splice donor signal, as found with ALV insertions at c-erbB and MuLV insertions at c-myb (Raines et al, 1985; Shen-Ong et al, 1986).

Enhancer insertion. Some ALV proviruses were found to be 3' to the c-myc gene or upstream and in the opposite orientation, and so could not direct c-myc transcription from viral promoters (Payne et al, 1982). In the mouse, similar modes of insertion have been found to be common for MuLV proviruses at the c-myc locus in thymic lymphomas (Corcoran et al, 1984; Selten et al, 1984), and for MMTV proviruses at the int-1 and -2 loci in mammary tumours (Nusse et al, 1984; Dickson et al, 1984). These findings suggested that enhancers in the proviral LTRs acting independently of orientation, could superimpose viral control of transcription from the normal cellular gene promoters.

Alteration of the product. Some insertions, such as IAP genes at the c-mos gene in murine plasmacytomas, or ALV proviruses at the c-erbB gene in chicken erythroleukaemias are within the coding domain of the c-onc gene suggesting that product truncation may be required for activation (Cohen et al, 1983; Nilsen et al, 1985). Also, point mutations have been detected in the coding region of a c-myc gene carrying an upstream ALV insertion (Westaway et al, 1984).

Amplification of the rearranged locus. It was found that some c-myc loci which carry REV or MuLV insertions were amplified to a small extent (Noori-Daloii et al, 1981; Li et al, 1984; Cuypers et al, 1986).

For mouse tumours, this appears to be due to duplication of the chromosome carrying the MuLV insertion (Cuypers et al, 1986).

Multiple insertions at different loci. There are a few examples of independent insertions at different cellular loci with oncogenic potential in the same tumour cell clone. These include MuLV proviruses at the c-myc and pim-1 loci (Selten et al, 1985) or at the Mlvi-1 and -2 loci (Tsichlis et al, 1985), or MMTV proviruses at the int-1 and -2 loci (Peters et al, 1986).

1.4 OTHER THEORIES OF RETROVIRUS-INDUCED ONCOGENESIS

Cell-derived oncogenes have not been found to be directly involved in all retrovirus-associated tumours, and some other oncogenic mechanisms have been proposed, as briefly outlined in the following:

1.4.1 Immunosuppression

Retroviral infection may be immunosuppressive to the host (Enrietto & Wyke, 1983; Jarrett, 1984). For example, young cats infected with FeLV often display immunosuppression and thymic atrophy (Anderson et al, 1971). This condition also precedes experimental induction of thymic lymphosarcoma by FeLV, suggesting that it predisposes the cat to neoplastic disease. One speculation might be that as the host produces more cells to compensate for lymphoid cell depletion there is an increased probability of occurrence of oncogenic mutations.

1.4.2 Chronic immunostimulation

In the mouse, the chronic immunostimulation theory of leukaemogenesis proposes that MuLV viraemia is essential to cause a chronic cellular immune reponse which is an important preceding step in T-cell leukaemogenesis (Ihle & Lee, 1982). This is thought to be mediated by production of interleukins by infected cells. The expanding pool of T-cells may be prone to acquiring oncogenic mutations perhaps by proviral insertions or other events.

1.4.3 Oncogenic potential of viral replicative genes

Viral replicative genes distinct from cell-derived oncogenes also may interfere with host cell growth. McGrath et al (1980) have proposed a model of receptor-mediated leukaemogenesis for murine thymic lymphomas where mitogenic stimulation of MuLV-infected T-cells occurs through binding of viral env products to specific antigen receptors. This model may apply to any retrovirus-associated lymphoma where the

tumour cells express functional antigen receptors.

Study of human T-lymphotropic viruses (HTLV-I and -II) and bovine leukaemia virus (BLV) has revealed the trans-activating tat genes, unrelated to previously known viral replicative genes, which augment viral transcription or the stability of viral transcripts through responsive elements in the LTR (Chen et al, 1986). A leukaemogenic role for tat genes has been proposed by Greene et al (1986) who presented evidence that tat products stimulate expression of the cellular IL-2 and IL-2 receptor genes, perhaps through control elements related to those in the viral LTR.

1.5 FeLV PATHOGENESIS

1.5.1 Infection

FeLV infects domestic cats and so provides an opportunity to study the role of a leukaemogenic retrovirus in naturally-occurring tumours. In 1973, it was demonstrated that FeLV can be transmitted horizontally, showing that retroviruses may be important agents of disease in an outbred population (Hardy et al, 1973). Transmission is usually horizontal through body fluids of infected cats, and may be congenital by virus transfer across the uterus to the embryo (Jarrett, 1984). Persistently viraemic cats show a high incidence of FeLV-related disease, although the outcome of infection depends on various factors such as virus dose and host age (Jarrett, 1984). A young cat encountering a high dose of virus is most susceptible whereas older cats are more resistant.

1.5.2 Neoplastic diseases

Spontaneous neoplastic diseases of the cat often involve haemopoietic tissue, and most are lymphoid in origin (Jarrett, 1984). The common tumours include thymic or multicentric lymphosarcomas which are usually virus-positive, and alimentary lymphosarcomas of which only one third are virus-positive. Some other forms of leukaemia may be under-diagnosed.

Experimental study of thymic lymphosarcoma development using the FeLV Rickard strain (Rickard et al, 1969) reveals early thymic atrophy followed by infiltration of the thymic remnant with malignant lymphocytes of unknown origin, which proliferate to form the tumour.

1.5.3 FeLV sub-groups

FeLV isolates may be grouped as sub-group A, B or C depending on properties of their envelope glycoproteins. FeLV-B and -C occur in nature only in association with FeLV-A which may reflect dependence on FeLV-A for transmission (Jarrett et al, 1978). FeLV-B and -C *env* genes are thought to result from recombination between a parental FeLV-A and endogenous proviral sequences (Stewart et al, 1986a; Neil & Onions, 1985). Different sub-groups may be associated with particular diseases, which may be due to different target cell preferences (Onions et al, 1982). Interestingly, FeLV-AB mixes may have a greater capacity to induce thymic lymphosarcoma than does FeLV-A alone (Jarrett, 1984).

1.5.4 Oncogenic mechanisms of FeLV

Direct oncogenic mechanisms involving FeLV include transduction and insertional mutagenesis of cellular oncogenes. Study of naturally-occurring feline tumours has revealed many examples of oncogene transduction by FeLV (Table 1.1). Initial studies focused on the feline sarcoma viruses, isolated from rare cases of multicentric fibrosarcoma in young cats, which were found to carry a variety of oncogenes (Besmer, 1983). Many of these v-*onc* genes were related to the tyrosine kinase gene family, but also included the *sis* and K-*ras* genes. Many of these viruses have been shown to induce sarcomas rapidly in young cats and to transform cells in culture.

However, in thymic lymphosarcomas, the most common tumour type associated with FeLV infection, it was more recently found that there is a relatively high incidence (~25%) of transduction of the *myc* gene (Neil et al, 1984; Levy et al, 1984; Mullins et al, 1984). This finding was important since it provided the first example of transduction of the mammalian *myc* gene, with v-*myc* genes previously only known in avian retroviruses. Secondly, since most of the tumours examined were field cases, it indicated that transduction may be more frequent in naturally-occurring tumours than commonly believed (Bishop, 1983). It also included T-cell lymphomas within the range of tumours associated with *myc* alterations; previously the gene was mainly associated with B-cell lymphomas of the chicken, man and mouse, and with various chicken leukaemias and carcinomas. Although FeLV/*myc* viruses have not been found to transform cells in culture, they induce thymic tumours with short latency in young cats (Neil et al, 1984; D.

Onions, G. Lees, D. Forrest & J. Neil, Int. J. Cancer, in press).

Apart from myc transduction, several field case and experimental lymphosarcomas showed alteration of the c-myc locus by proviral insertion, amplification or other rearrangement (Neil et al, 1984; D. Forrest et al, submitted; J. Mullins, pers. comm.). In experimentally-induced tumours, proviral insertions occur at a similar high frequency (≥50%) to that recorded in MuLV-induced thymic lymphomas of the mouse (Selten et al, 1984; O'Donnell et al, 1985).

A detailed study of the normal and altered myc genes in feline leukaemias is the main subject of this thesis, and so a brief review concerning the normal and oncogenic roles of the myc gene now follows.

1.6 THE c-myc GENE

1.6.1 General

The c-myc gene probably has a critical role in control of normal and tumour cell growth, although its precise function remains unknown at present. First, since the gene is highly related between species (van Beveren et al, 1985) it is presumed to play a fundamental role. Secondly, c-myc expression generally correlates with growth of various cell types (see below) and appears to be subject to complex controls, suggesting that the gene serves a critical role requiring fine regulation (Saito et al, 1983; Dani et al 1984; Remmers et al, 1986; Bentley & Groudine, 1986a). Thirdly, alteration of the gene by diverse mechanisms has been associated with a variety of tumours (Table 1.2). This implies that the gene has a crucial role in normal growth control and is an important target in oncogenesis.

1.6.2 The c-myc gene in cell growth

Expression of the c-myc gene is seen in many normal and tumour tissues (Gonda et al, 1982; Slamon et al, 1984). Generally, expression correlates with growth, being induced upon mitogenic stimulation of resting normal cells and tissue (Kelly et al, 1983; Makino et al, 1984), and down-regulated during growth inhibition of haemopoietic and other cells (Reitsma et al, 1983; Lachman & Skoultchi, 1984; Campisi et al, 1984). In growing cells, c-myc RNA and protein are expressed continuously throughout the cell cycle (Thompson et al, 1985; Hann et al, 1985).

The myc product may enable cells to enter and progress through the cell-cycle. Resting BALB/c 3T3 fibroblasts require "competence" factors

such as PDGF to become sensitive to later-acting factors such as epidermal growth factor, which are necessary for progression through DNA synthesis and mitosis (Stiles et al, 1979). Direct evidence that c-myc is a competence factor comes from micro-injection of the c-myc protein into the nuclei of fibroblasts which promotes DNA synthesis without requiring PDGF (Kaczmarek et al, 1985).

It is of interest in studying the myc gene in feline T-cell tumours that similar events occur in lymphoid cell growth. Stimulation of resting lymphocytes with mitogens, or specific stimulation of the T-cell antigen receptor (TCR) by monoclonal antibody rapidly induces c-myc expression (Kelly et al, 1983; Reed et al, 1985). Normally, proliferation of mature T-cells in the immune response responds to antigenic stimulation mediated by the TCR, analogously to fibroblast proliferation through growth factor receptors (Smith, 1985). Immunostimulation of T-cells induces an IL-2 responsive state for IL-2 dependent DNA synthesis and mitosis (Waldman, 1986). Hence, c-myc expression may be a common factor which primes T-cells and fibroblasts for growth.

How might the myc product mediate growth control? The myc product was found to be located in the nucleus which suggested a possible role in interaction with nucleic acids, possibly in controlling expression of other genes. In vitro studies showed that the myc product binds DNA (Bunte et al, 1984; Watt et al, 1985), perhaps a function of the C-terminus which is rich in basic amino acids. Whether DNA binding occurs in vivo is not known, but a possible role for c-myc in controlling gene expression in trans was shown by co-transfection studies with genes under control of heat shock gene promoters (Kingston et al, 1984).

1.6.3 Oncogenic potential

The oncogenic activity of the myc gene is known principally from study of avian myc-transducing viruses which induce a range of carcinomas and leukaemias in chickens (Hayman, 1983). Recent studies have shown that myc-transducing FeLVs rapidly induce thymic lymphosarcomas in cats (D. Onions et al, in press).

There is some evidence that rearranged c-myc genes may have transforming potential, based on introduction of c-myc constructs into cells in culture or into transgenic mice. In summary, the in vitro

studies indicate that the gene may not always be active on its own in transforming fibroblasts, but it does transform efficiently in cooperation with ras genes (Keath et al, 1984; Lee et al, 1985; Martin et al, 1986). Experiments with transgenic mice show that tumours reproducibly arise in tissues in which the c-myc construct is expressed (Stewart et al, 1984b; Adams et al, 1985).

In almost all tumours in which a rearranged c-myc gene has been identified, it remains to be demonstrated that the gene has transforming activity. However, since v-myc genes and c-myc constructs display transforming activity, then by inference c-myc rearrangements may have had a similar role in the tumours in which they were identified. The frequency of c-myc rearrangement in tumour development may also support this view. For example, > 80% of ALV-induced chicken bursal lymphomas carry a proviral insertion and most human Burkitt's lymphomas carry a chromosomal translocation breakpoint at the c-myc locus (Crittenden & Kung,1984; Leder et al, 1983).

1.7 EXPERIMENTAL OUTLINE

I began my study a short time after the discovery that the myc gene was either transduced by FeLV or otherwise rearranged in a subset of feline leukaemias (Neil et al, 1984; Levy et al, 1984; Mullins et al,1984). This finding prompted two main lines of research in Glasgow into the molecular mechanisms underlying feline leukaemias. First, what are the mechanisms of alteration of the myc gene in tumours? Secondly, since the myc gene was only implicated in ~30% of tumours, what other events may be involved?

My work involved isolation and characterisation of a clone of the normal feline c-myc gene. This was necessary since it was not known how the feline gene compared with that in other species. Also, analysis of the sequence of the c-myc gene was essential for investigation of possible structural changes present in FeLV v-myc genes (Chapter 3). Determination of the normal c-myc gene structure also permitted detailed mapping by Southern blot hybridisation analysis of rearrangements of the c-myc gene in other tumours, since little was known of the nature of these alterations (Chapter 4).

It was also important to study, by Northern blot and S1 nuclease analysis, expression of the normal, rearranged and transduced myc genes

to understand the consequences of structural alteration (Chapters 5 and 6).

Finally, the available tumours were characterised with respect to expression of transcripts of the T-cell antigen receptor to provide clues to other events important in development of these leukaemias (Chapter 7).

Cellular gene	Associated disease	Number of isolates	Refs.
fes	fibrosarcoma	3	1
fms	"	2	1,2
abl	"	1	1
sis	"	1	1
kit	"	1	3
fgr + actin	"	1	4
K-*ras*	"	1	1
myc	lymphosarcoma	8	5,6
tcr	"	1	6

Table 1.1. Cellular genes transduced by feline retroviruses.

Refs. 1, review, Besmer, 1983; 2, Besmer et al, 1986a; 3, Besmer et al, 1986b; 4, Naharro et al, 1984; 5, Neil et al, 1984; Levy et al, 1984; Mullins et al, 1984; 6, R. Fulton, D. Forrest, R. MacFarlane, D. Onions and J. Neil, Nature, in press.

Mechanism of c-myc Alteration	Species	Associated Tumours	Refs.
Transduction	chicken	Leukaemia, Lymphoma Carcinoma	1
	cat	T cell lymphoma	2
Retroviral insertion	chicken	B cell lymphoma	3,4
	mouse	T cell lymphoma	5,6,
	cat	" "	7
	rat	" "	8
Other insertion	mouse	Plasmacytoma (IAP gene)	9
	dog	Transmissible venereal tumour (Transposon)	10
Chromosomal translocation	man	B cell lymphomas	11,12
		T cell "	13,14
		Renal cell carcinoma	15
	mouse	Plasmacytoma	12
	rat	Immunocytoma	16
Amplification	man	Lung carcinoma	17
		Colon "	18
		Gastric "	19
		Myeloid leukaemia	20
		Glioblastoma	21
	cat	Spleen lymphoma	2
		T cell "	2
Point mutations in: coding regions	man	B cell lymphoma	22,23,24
	chicken	" "	25
non-coding regions	man	B cell lymphoma	26,27
	mouse	Plasmacytoma	28

REFERENCES: 1, Hayman, 1983; 2, Neil et al, 1984; 3, Hayward et al, 1981; 4, Noorii-Daloii et al, 1981; 5, Corcoran et al, 1984; 6, Selten et al,1984; 7, D. Forrest et al, submitted; 8, Steffen, 1984; 9, Greenberg et al, 1985; 10, Katzir et al, 1985; 11, Leder et al, 1983; 12, Marshall, 1985; 13, Erikson et al, 1986; 14, Shima et al, 1986; 15, Drabkin et al, 1985; 16, Sumegi et al, 1983; 17, Little et al, 1983; 18, Alitalo et al, 1983; 19, Shibuya et al, 1983; 20, Collins & Groudine, 1982; 21, Trent et al, 1986; 22, Rabbitts et al, 1984; 23, Showe et al, 1985; 24, Care et al, 1986; 25, Westaway et al, 1984; 26, Battey et al, 1983; 27, Wiman et al, 1984; 28, Stanton et al, 1984. (Referencing has been selective).

Table 1.2. Mechanisms of tumour-specific alteration of the c-myc gene in different species.

CHAPTER TWO

MATERIALS AND METHODS

Part A. MATERIALS

2.1 Molecular clones

Recombinant plasmids and bacteriophage clones used in this study are listed in Table 2.1. These clones were stored in TE buffer (10mM Tris.HCl pH 8.0, 0.1mM EDTA) at -20°C. Clones were propagated in the following *E. coli* host strains: pBR322-based clones in strain HB101; pUC8-based clones in strain JM83; bacteriophage λ clones in strain LE392 and M13 phage clones in *E. coli* strain JM103.

2.2 Chemicals, enzymes and other materials

Most chemicals and enzymes and other materials were obtained from The Sigma Chemical Company, BDH Chemicals, The Boehringer Corporation, BRL Inc., Difco Laboratories, Gibco and Pharmacia with the following exceptions: bacterial alkaline phosphatase from Worthington [further purified according to Hall (1981)]; collodion dialysis bags from Sartorius; GeneScreen membranes from NEN; X-omat x-ray film from Kodak.

2.3 Media and Antibiotics

L-broth : 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride. Ampicillin was included when required at 100µg/ml.

L-agar : L-broth containing 1.5% (w/v) agar.

Top agar/x-gal: L-agar containing 100µg/ml ampicillin and 250µg/ml x-gal.

YT medium and agar and top agar for M13 cloning were prepared as described in the Amersham M13 cloning and sequencing handbook.

2.4 Radiolabelled nucleotides (obtained from Amersham)

Type	Specific activity (Ci/mmol)	Code
[α-^{32}P]dATP	3000	PB10204
[α-^{32}P]dCTP	"	PB10205
[α-^{32}P]dGTP	"	PB10206
[α-^{32}P]dTTP	"	PB10205
[γ-^{32}P]ATP	6000	PB10218
[^{35}S]dATPαS	600	SJ304

2.5 Biological samples

All tumour and normal tissues and cultured cells were obtained from the Glasgow University Veterinary School. I occa sionally grew tumour cell lines obtained from the same source (section 2.2.12). Tumours analysed in this study are listed in Table 2.2. Several RNA and genomic DNA samples had already been prepared by R. McFarlane and G. Lees.

Part B. GENERAL METHODS

2.6 Restriction enzyme digestion of DNA.

Digests were performed in sterile 1.5ml Eppendorf microcentrifuge tubes according to the enzyme supplier's specifications in the recommended buffers.

2.7 Ethanol precipitation of nucleic acids.

Generally, aqueous DNA and RNA samples were precipitated with 1/10 volume of 3M sodium acetate and 2.5 volumes of ethanol at -70°C for 0.5h, or -20°C for >2h. Samples were centrifuged at 9950g for 10 minutes. The pellet was washed at least once with 80% ethanol. Genomic DNA samples were precipitated with 0.5 volume of 7.5M ammonium acetate and 2 volumes of this total aqueous volume of ethanol at -20°C for >2h.

2.8 Phenol extraction.

Residual agarose was removed from gel-purified DNA fragments by at least four phenol extractions using standard methods [Maniatis et al (1982)] and protein was removed from DNA samples with two or three extractions. When required, the resulting aqueous solution was reduced in volume by isobutanol extraction for ease of precipitation.

2.9 Gel electrophoresis.

In general, agarose gels were used for separation of nucleic acids >1kb in size and polyacrylamide gels for DNA fragments <1kb in size.

(A) AGAROSE GEL ELECTROPHORESIS

Electrophoresis buffers (1X):

(i) TEA buffer: 40mM Tris.HCl pH8.15, 20mM sodium acetate, 20mM sodium chloride, 2mM EDTA.

(ii) TBE buffer: 100mM Tris borate, 83mM boric acid, 1mM EDTA pH8.0.

(iii) MOPS buffer: 20mM MOPS pH7.0, 5mM potassium acetate, 1mM EDTA.

Sample loading solutions:

(i) 10X Sample loading solution for all gels (except where stated): 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 25% (w/v) Ficoll-400 in distilled water.

(ii) Northern blot analysis RNA loading buffer: 50% (v/v) formamide, 2.2M formaldehyde, 1X MOPS buffer.

(iii) Formamide/dye loading solution for denaturing acrylamide gels: 99% formamide/1% xylene cyanol.

Notes:

(i) During electrophoresis of gels with TEA buffer, the buffer was circulated using a peristaltic pump.

(ii) When required, after electrophoresis, gels were stained in ethidium bromide (10μg/ml) for 0.5h then nucleic acids visualised using U.V illumination, and photography with a polaroid camera.

(iii) All acrylamide gel mixtures were filtered through Whatman no.1 filter paper using a Buchner flask prior to polymerisation.

Minigels. 1% (w/v) agarose gels (13 x 9.5 x 0.5 cm) with TEA buffer were used to analyse restriction enzyme digests of DNA. 1% (w/v) agarose gels with TBE buffer were used to check recovery of M13 phage template DNA and to check that total cellular RNA preparations were not degraded. Samples containing 1/10 volume of 10X loading solution were applied (~1-2μg of DNA or ~4μg of RNA per lane) and electrophoresed at 40V for 2-4h when using TEA buffer, or 100V for 2h with TBE buffer.

Agarose gels for Southern blot analysis. Restriction enzyme digests of genomic DNA (20μg per lane) were electrophoresed in 0.6% (w/v) agarose gels (19.5 x 14.5 x 0.5 cm) with TEA buffer at 25V overnight.

Preparative agarose gels. Required DNA fragments were isolated from DNA restriction digests by electrophoresis through typically, 1.2% (w/v) low melting point agarose gels (19.5 x 14.5 x 0.5 cm) with TEA buffer at 30-35V overnight at 4°C.

Agarose gels containing formaldehyde for Northern blot analysis.1% (w/v) agarose gels (19.5 x 14.5 x 0.5 cm) were prepared by dissolving 2g of agar in 147ml of water in a microwave oven. When cooled to 60°C, 33ml of formaldehyde and 20ml of 10X MOPS buffer were added, and the gel poured. RNA samples were freeze-dried, redissolved in 20μl of RNA loading buffer, loaded and electrophoresed at 120V for 2-4h using 1X

MOPS buffer.

(B) POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Generally, 4% polyacrylamide gels (16.0 x 16.0 cm x 1.5 mm) were made as a 50ml stock solution containing 4% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 1X TBE, 1% (w/v) ammonium persulphate (APS) and 30µl of TEMED (Maniatis et al, 1982). 20µl samples (1-2µg of DNA) were loaded per lane and electrophoresed using 1X TBE buffer at 200V for 2-3h.

Denaturing PAGE. Polyacrylamide gels (40 x 20 cm x 0.3 mm) containing 7M urea were used to fractionate products of DNA sequencing reactions and of RNA S1 nuclease analyses. Except where stated, 6% acrylamide gels were used, which were made as a 60ml stock solution containing 6% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 1X TBE, 0.8% (w/v) APS, 25.2g of urea and 24µl of TEMED. Samples were redissolved in formamide/dye solution. Gels were electrophoresed at 1200-1500V for 15 minutes prior to loading samples, and samples were electrophoresed at the same voltage for the appropriate time (e.g 1.5-4h) using 1X TBE buffer.

2.10 Isolation of DNA fragments from preparative agarose gels.

The band of interest was located on the gel, excised with a scalpel, placed in a 50ml polypropylene tube (Falcon) with 5ml of distilled water and melted in a 70°C water bath for 10 minutes. The DNA was then purified by at least four phenol extractions and ethanol precipitation.

2.11 Isolation of DNA fragments by preparative PAGE.

The band of interest was excised from the gel with a scalpel, crushed, and the DNA eluted into a solution of 500mM ammonium acetate, 10mM magnesium acetate, 0.1 mM EDTA and 0.1% SDS by overnight incubation at 37°C (Maxam & Gilbert, 1980). The aqueous solution was collected through siliconised glass wool and the DNA purified by ethanol precipitation.

2.12 Hybridisation analysis of DNA and RNA.

Hybridisation analyses were performed essentially as described in the GeneScreen hybridisation instruction manual (NEN, Boston).

Solutions:

50X Denhardt's solution: 1% (w/v) of each of Ficoll-400, polyvinylpyrrolidone and BSA (Pentax fraction V).

20X SSC: 3M sodium chloride and 0.3M sodium citrate.

20X phosphate buffer: 0.5M Na_2HPO_4/ 0.5M NaH_2PO_4 pH6.5.

Southern blot transfer of DNA. DNA samples were electrophoresed and transferred by the Southern (1975) method onto GeneScreen membranes. This involved denaturation of the DNA in the gel in 1.5M NaCl/0.5M NaOH, followed by neutralisation in 3M NaCl/0.5M Tris.HCl pH7.0, then washing for 1h with 1X phosphate buffer. Transfer was performed overnight (> 14h) in 1X phosphate buffer. The membrane was then rinsed in 1X phosphate buffer and baked at 80°C for 2-4h to fix the DNA.

Northern blot transfer of RNA. This procedure was the same as for DNA transfer except that the denaturation and neutralisation steps were unnecessary, and transfer was continued for the longer time of >18h as this improves transfer of larger RNA molecules (Maniatis et al, 1982).

Hybridisation procedure. Standard high stringency conditions for analysis of feline nucleic acids with probes of feline origin (when using a probe derived from a different species, stringency was reduced as necessary): membranes were pre-wetted in 1% Triton-X100, then prehybridised in sealed polythene bags in a 10ml volume containing: 50% (v/v) formamide (de-ionised), 5X SSC, 5X Denhardts solution, 1X phosphate buffer, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS and 100µg/ml of denatured salmon sperm DNA, at 42°C overnight with agitation. Then radioactive probe was added to a concentration of 5ng/ml with a further 2ml of prehybridistion solution and hybridised overnight at 42°C with agitation. The next day membranes were rinsed in 2X SSC several times at room temperature, then washed at 60°C with agitation in 0.1X SSC/0.5% SDS, for 1h, including three changes of wash solution. Membranes were rinsed in 0.1X SSC at room temperature for several minutes, dried on Whatman 3MM paper and sealed in thin polythene bags for autoradiography.

2.13 Preparation of radioactively-labelled DNA fragments

Nick translation. DNA inserts for use as hybridisation probes were gel-purified from their vectors and radioactively-labelled using a kit provided by Amersham (code N.5000), which contained necessary enzymes

(DNAseI and DNA polymeraseI) and buffers. Generally, 0.5μg of DNA was made to 45μl with 10μl of buffer (containing non-radioactive dATP, dGTP, dTTP), 100μCi of radiolabelled dCTP and distilled water. Then 5μl of enzyme solution was added, mixed gently and the mixture incubated at 14°C for 2.5h. Unincorporated nucleotides were removed by gel-filtration through a Sephadex G50 column, eluted with 5mM Tris.HCl pH8.0/1mM EDTA/0.1% (w/v) SDS. Progress of the labelled DNA peak was monitored and peak fractions were collected. The specific activity of the labelled DNA was calculated (usually 1-2 x 10^8 cpm/μg) and the appropriate amount of labelled DNA was used immediately as hybridisation probe.

5' end-labelling. This procedure was used to radiolabel DNA fragments for use as molecular size markers, for use as S1 nuclease mapping probes (Chapter 5) or for use as substrates for the chemical cleavage sequencing method. The DNA fragment (1-5μg) with overhanging 5' ends or blunt ends was first dissolved in 90μl of TE pH8.0 and mixed with 10μl of bacterial alkaline phosphatase. The reaction was incubated at 37°C for 1.5h, then extracted with phenol and purified by ethanol precipitation. The DNA pellet was dissolved in 6μl of water, 2.4μl of 10X kinase buffer (0.1M magnesium chloride, 0.5M Tris.HCl pH8.0), 1.2μl of 100mM DTT and 0.5μl of 10mM spermidine. Then 12μl (120μCi) of [γ-^{32}P]ATP was added with 5 units of polynucleotide kinase to a total volume of 24μl. This was incubated at 37°C for 0.5h. The reaction was phenol extracted and unincorporated nucleotides removed by dialysis in a micro-collodion bag against TE pH8.0, or by gel-filtration through a Sephadex G50 column.

3'end-labelling. This procedure was used to radiolabel HindIII-digested λDNA size markers and to make S1 nuclease mapping probes (Chapter 5) by template-dependent extension of recessed 3' ends using DNA polymerase I Klenow fragment. 1-2 μg of DNA was mixed with 5μl of buffer solution from a nick translation kit, 10μl of [α-^{32}P]dCTP, 0.5μl of 100mM spermidine and 10 units of enzyme in a volume of 50μl. The reaction was incubated at room temperature for 5 minutes, then 1 μl of chase solution (non-radioactive 100mM dCTP) was added and incubation continued for a further 25 minutes. The reaction was phenol extracted and unincorporated nucleotides removed as described above. End-labelled probes could be stored at -20°C.

2.14 Plasmid cloning

All recombinant plasmids made in this study were constructed in pUC8 using ampicillin and colour selection for recombinant clones.

Solutions: 10X ligase buffer: 0.5M Tris.HCl pH7.6, 0.08M $MgCl_2$.
10X DNA transformation buffer: 10mM of each of $MgCl_2$, $CaCl_2$ and Tris.HCl pH7.5.

Ligation. Vector and insert DNAs digested with appropriate enzymes were phenol extracted and ethanol precipitated. In general, insert DNA was added in 10X molar excess to 50ng of pUC8 in a 10µl volume containing 1X ligase buffer, 10mM ATP, 10mM DTT and 6 units of T4 DNA ligase and incubated overnight at 14°C.

Transformation. Competent E. coli strain JM83 were prepared according to Hanahan (1983). Half of the ligation reaction was made to a 100µl volume containing 1X transformation buffer, mixed with 100µl of competent cells and left on ice for 0.5h. The mixture was heat-shocked at 37°C for 5 minutes and transferred to 3ml of L-broth at 37°C for 1.5h. Ampicillin (100µg/ml) was added and the mixture plated out at low and high densty on L-agar plates with a top agar/x-gal layer containing ampicillin (section 2.1.3). Plates were incubated overnight at 37°C. Several positive (white) colonies were picked, grown in small (10ml) cultures and plasmid DNA isolated (Maniatis et al, 1982) and digested with restriction enzymes to check that the plasmid represented the expected clone.

2.15 Nucleic acid preparations.

Guanidinium thiocyanate solution: 5M guanidinium thiocyanate, 50mM Tris.HCl pH7.0, 50mM EDTA, 5% (v/v) β-mercaptoethanol.

Large scale preparation of plasmid DNA. Bulk preparation of plasmids followed the bacterial growth and alkaline lysis procedures given in Maniatis et al (1982) with final plasmid purification by centrifugation through CsCl/ethidium bromide gradients.

Feline genomic DNA. Liquid nitrogen frozen tissue was crushed then lysed in 5-10ml of guanidinium thiocyanate solution with 8-10 strokes of a loose fitting pestle in a Dounce homogeniser. Tissue culture cells were washed in phosphate buffered saline and lysed in guanidinium thiocyanate by pipette action. Sodium acetate was added to 20mM, then the DNA precipitated with an equal volume of isopropanol and spooled

onto a glass rod. The DNA was washed in 70% and 100% ethanol, dried, and redissolved in 5ml of 10mM of each of NaCl, EDTA, Tris.HCl pH8.0 and 0.5% (w/v) SDS. Proteinase K (50μg/ml) was added and the mixture incubated overnight at 37°C, phenol extracted and dialysed against TE pH8.0. RNAse A (50μg/ml) was added and the solution incubated for 0.5h at 37°C, phenol extracted and dialysed as before. The DNA could then be stored at 4°C.

Feline total cellular RNA. Tissue samples or cells were lysed as for DNA preparation. Total cellular RNA was isolated by centrifugation at 210,000g for 24h at 15°C through 5.7M CsCl as described in Chirgwin et al (1979). The RNA pellets were ethanol precipitated, redissolved in DEP-treated water and stored at -70°C.

2.16 DNA sequencing.

The M13 chain-termination method of Sanger et al (1980) and the method of chemical-cleavage of end-labelled fragments by Maxam and Gilbert (1980) were used in this study. Both methods were performed according to well-established protocols which were followed closely and will not be reiterated here. The protocol for M13 cloning and sequencing was described in the Amersham handbook, and made use of mp10 and mp11 vectors, and the chemical-cleavage method was described in Maxam and Gilbert (1980) with modifications in Stewart (1983) (Table 2.3). In all cases the substrates for chemical-cleavage sequencing were single strands of DNA which were prepared as described in Chapter 5. The M13 method was preferentially used but occa sional short GC-rich sequences did not yield clear results with this method and the chemical-cleavage method was used to confirm these sequences.

2.17 Tissue culture

Suspension cultures of leukaemic feline cell lines F422 and T3 (D. Onions et al, submitted) were grown in 50% Liebovitz L15/50% McCoys 5A medium supplemented with 2mM L-glutamine, 10% foetal bovine serum and 400 units/ml of penicillin/streptomycin, to 5×10^5 - 2×10^6 cells/ml and were sub-cultured twice weekly.

CLONE	INSERT	SPECIES OF ORIGIN	VECTOR	REFS. AND NOTES
pCT4P1	v-myc	cat	pUC8	1
pCT8K0	FeLV U3 FeLV U5	cat	pAT153	2
λSPF62	c-myc	cat	λJ1	3
λCM-1	c-myc	cat	λgt.WES	4
pFMC-1	c-myc	cat	pUC8	4
pSB1.8	c-myc	cat	pUC8	4
pS700	c-myc	cat	pUC8	4
pMC41HE	c-myc	man	pBR322	5
pJα2	α-TCR	man	pBR322	6
pB400	β-TCR	man	pUC8	6
Pim-1	pim-1	mouse	pBR322	7

Table 2.1 Molecular clones used in this study.

Refs. and notes:
1, Neil et al (1984); 2, provided by J. Neil; 3, Mullins et al (1984); 4, clones generated in this study; 5, Dalla-Favera et al (1982); 6, Collins et al (1985); 7, Cuypers et al (1984).
All plasmid clones were grown using ampicillin selection.

Tumour case	Lymphosarcoma type	FeLV +/-	*myc* alteration
84793	Thymic	+	V
84929	Thymic	+	V
F422*	Thymic	+	V
T3	Thymic	+	V
T11	Multicentric	+	V
T17	Thymic	note 1	V
T5	Thymic	+	R, A
T7	Thymic	+	R
T8	Thymic	+	R
T24	Thymic	+	R
T9	Splenic	-	A
T10	Thymic	+	-
T14	Thymic	+	-
T18	Thymic	-	-
T19	Thymic	+	-
T21	Thymic	-	-
T23	Thymic	-	-
84904	Thymic	+	-
86503	Thymic	-	-
86800	Thymic	+	-
87416	Alimentary	+	-
89407	Thymic	+	-
89960	Thymic	-	-
FL74*	note 2	+	-

Table 2.2. Tumours analysed in this study.

* Denotes tumour cell line; all other cases were primary tumours. All tumours were field cases except T5, T8, T10 and T24 which were experimentally induced (Chapter 4).

FeLV status was determined by virus isolation assay (Jarrett et al, 1982) and by hybridisation analysis of integrated proviruses using a U3 probe specific for exogenous FeLV (Casey et al, 1981).

Notes:

1. Serologically, T17 was FeLV (-), but tumour DNA was shown to contain FeLV proviruses (R. Fulton et al, in press).
2. The leukaemic FL74 line was established from a tumour in a kidney which had the erythrocyte-rossetting property characteristic of lymphoid cells (Theilen et al, 1969; D. Onions, pers. comm.).

Key to *myc* alterations: V, FeLV/*myc* provirus; R, rearranged c-*myc* gene; A, amplification of the c-*myc* locus.

	G	G + A	C + T	C
1.	1µl carrier DNA 1µl ^{32}P-DNA 98µl DMS buffer	1µl carrier DNA 1µl ^{32}P-DNA 11µl H_2O	1µl carrier DNA 1µl ^{32}P-DNA 6µl H_2O	1µl carrier DNA 1µl ^{32}P-DNA 8µl sat. NaCl.
2.	0.5µl DMS	2.5µl pyr. form.	15µl Hz.	15µl Hz.
3.	20°C, 4mins.	30°C, 70mins.	20°C, 6mins.	20°C, 8mins.
4.	24µl DMS stop 400µl Ar. ETOH	freeze -70°C lyophilise. add 10µl H_2O freeze -70°C lyophilise.	60µl Hz. stop 250µl Ar. ETOH	
5.	-70°C, 15mins. c'fuge (9950g) 5mins.		-70°C, 15mins. c'fuge (9950g) 5mins.	
6.	60µl 0.3M Na.Ac. 200µl Ar. ETOH		60µl 0.3M Na.Ac. 200µl Ar. ETOH	
7.	repeat step 5		repeat step 5	
8.	200µl 70% ETOH		200µl 70% ETOH	
9.	repeat step 5		repeat step 5	
10.	200µl 70% ETOH		200µl 70% ETOH	
11.	repeat step 5		repeat step 5	
12.	dry pellet		dry pellet	
13.	100µl 1M Piperidine			
14.	90°C, 30mins.			
15.	c'fuge (9950g) 30secs.			
16.	freeze -70°C, lyophilise, add 20µl H_2O, freeze -70°C, lyophilise, add 20µl H_2O, freeze -70°C, lyophilise.			

Table 2.3. Protocol used for DNA sequencing by the Maxam and Gilbert method, modified by Stewart (1983).

Abbreviations: DMS, dimethylsulphate; Hz, hydrazine; sat. NaCl, saturated NaCl; pyr. form., pyridine formate, pH2; Ar. EtOH; Analar ethanol; Na.Ac., sodium acetate.

All reactions were performed in 1.5ml siliconised Eppendorf tubes. DMS and Hz wastes were disposed of into 5M NaCl and 3M ferric chloride respectively and steps involving handling of these chemicals were performed in a fume hood. After final lyophilisation at step 16, the radioactivity of each sample was determined by Cerenkov counting.

CHAPTER THREE

THE FELINE c-myc GENE: STRUCTURE AND RELATIONSHIP TO FELINE v-myc GENES.

3.1 INTRODUCTION

The discovery of transduced v-myc and rearranged c-myc genes in feline leukaemias prompted a detailed characterisation of the feline c-myc gene. This was necessary so that the altered myc genes could be compared to the normal c-myc structure with the aim of understanding what changes may lead to oncogenic activation of the gene.

When this study began in late 1983, reports on the structure of the human, mouse and chicken c-myc genes showed that a highly related coding domain (of two exons, later designated as exons 2 and 3) was present in each of these species, which was a typical feature of the cellular progenitor sequences of a transduced v-onc gene (Watt et al, 1983a; Gazin et al, 1983; Bernard et al, 1983; Watson et al, 1983). The human and murine genes showed most similarity, and they also possessed a common upstream exon (exon 1) with some puzzling features. It formed part of the mRNA but apparently was non-coding. There were also two distinct promoter-like elements (P1 and P2) at the 5' end of this exon (Watt et al, 1983b; Battey et al, 1983). The significance of this structure was unknown but it suggested that expression of the gene may be subject to complex controls (Saito et al, 1983).

Preliminary Southern blot hybridisation analysis indicated that the feline c-myc gene was related to the gene in other species at least in coding exons 2 and 3 (Neil et al, 1984; Mullins et al, 1984). However, detailed comparison required isolation and characterisation of a molecular clone of the gene, which should also reveal whether an exon 1 domain was present. The clone would be important to allow sequence comparison with transduced feline v-myc genes to investigate what structural changes might play a role in oncogenic activation of the myc gene. The clone would also provide probes to map in detail c-myc rearrangements in tumours (Chapter 4) and to analyse c-myc transcripts in normal and tumour cells (Chapter 5).

3.2 MATERIALS AND METHODS

All procedures were as described in Chapter 2 except those dealing

with isolation of a clone of the feline c-myc gene from a bacteriophage λ library which were described in Maniatis et al (1982).

Media. LAM broth : L-broth containing 10mM magnesium sulphate and 0.2% (w/v) maltose.

LAM agar : LAM broth containing 1.5% (w/v) agar.

LAM agarose: LAM broth containing 0.6% (w/v) agarose.

Outline. Previous Southern blot analysis of feline genomic DNA indicated that the c-myc gene was present on a 10 kb EcoRI fragment (Neil et al, 1984). Hence, an available, unamplified λgtWES library of 500,000 clones containing EcoRI-digested, 8-15kb size-selected genomic DNA from normal feline embryonic fibroblasts of the FEA strain (Jarrett et al, 1973) was screened by the plaque hybridisation procedure (Benton & Davis, 1977) using a probe derived from a feline v-myc clone (pCT4/P1; Neil et al, 1984).

Procedures. Positive plaques were identified as described in Maniatis et al (1982). Hybridisation was performed as described in Chapter 2 except that no dextran sulphate was used and the membranes were contained in polythene boxes. Duplicate phage impressions were analysed in the first round of screening to confirm positive plaques. Positive plaques were picked, and the titre of plaque forming units determined using E.coli strain LE392. Recombinant phage were replated in LAM agarose top layer on LAM agar for the next round of plaque purification. This procedure was repeated until well-separated plaques could be picked.

DNA was prepared from a single positive bacteriophage clone by a liquid lysis method. 2×10^8 bacteria were infected with $\sim 10^6$ pfu of the bacteriophage suspension and were then transferred into LAM broth for overnight growth and lysis at 37°C with agitation. The lysate supernatant was collected and treated with DNAseI (10μg/ml) overnight at room temperature. 1/3 volume of 1.5% (w/v) SDS, 0.3M Tris.HCl pH9.0, 0.15M EDTA was added and the solution incubated at 70°C for 0.5h. Then 1/6 volume of 8M potassium acetate was added and the solution placed on ice for 0.5h. The supernatant was collected and precipitated with 3/5 volume of isopropanol at room temperature for 0.25h. The pellet was collected, dried, redissolved in water and treated with RNAse A (50μg/ml) at 37°C for 0.5h. The λ DNA was precipitated, dried and redissolved in TE pH8.0 and stored at 4°C.

3.3 RESULTS AND DISCUSSION

3.3.1 Isolation of clones of the normal feline c-myc gene.

Three myc-hybridising plaques identified during first round screening were picked for plaque purification. Unfortunately, one plaque did not yield further positive plaques. The other two (λCM-1 and -2) yielded well-separated, positive plaques from which the bacteriophage DNA was purified. The 10 kb inserts were isolated and sub-cloned into pUC8 to generate plasmids pFMC-1 and -2. The restriction map of pFMC-1 was determined using single and double enzyme digests, and agreed with the tentative map determined by hybridisation analysis of genomic DNA (Fig.3.1).

3.3.2 Location of exons on the c-myc clone.

Exons 2 and 3: Duplicate Southern blots of pFMC-1 digests were analysed with human exon 2 and 3-specific probes (Table 3.1; Fig.3.2). The exon 2 probe hybridised to fragments from the region expected according to the preliminary map. Thus, a 500 bp PstI band, a 2.0 kb BglII band and a 2.5 kb BamHI band located the exon 6.5-8.0 kb from the 5' end of the insert (Fig.3.1). The exon 3 probe hybridised to fragments from the predicted exon 3 region, notably a 1.3 kb BamHI band and a 2.0 kb PstI band derived from the extreme 3' end of the pFMC-1 insert. Subsequent sequence analysis showed the 3' EcoRI site of the cloned insert to be 3' to the exon 3 translational stop codon but 5' to the putative polyadenylation signals (Stewart et al, 1986b). The locations for exons 2 and 3 agree with those of other workers who have isolated feline c-myc clones (Mullins et al, 1984; Soe & Roy-Burman, 1984).

Several bands not from the expected region also hybridised to the exon 3 probe. However, these bands did not prevent assignment of the exon 3 location shown, as some coincide with exon 2 region fragments. This can be explained by a short exon 2 sequence at the 5' end of the v-myc-derived probe used. The remaining unexpected bands result from limited hybridisation to vector fragments (e.g. EcoRI digestion yielded a hybridising band for the expected 10 kb insert but also a fainter 2.7 kb linearised pUC8 band). The positions of exons 2 and 3 have been confirmed by sequence analysis by colleagues in Glasgow (Stewart et al, 1986b).

Exon 1: The location of exon 1 was not known, since a human exon 1-specific probe failed to give a hybridisation signal with feline genomic DNA even under conditions of reduced stringency. However, the probe detected bands in digests of the pFMC-1 clone using low stringency hybridisation conditions (Table 3.1; Fig.3.3). The bands detected, including a 2.3 kb BglII band and a 1.8 kb SalI/BamHI band, located exon 1 4.7-6.5 kb from the 5' end of the pFMC-1 insert. To facilitate sequence analysis, the SalI/BamHI 1.8 kb fragment was sub-cloned into pUC8 to generate the plasmid pSB1.8. Hybridisation analysis of digests of pSB1.8 confirmed that it contained exon 1, approximately in the middle of the insert (Fig.3.3).

Soe and Roy-Burman (1984) had previously reported the location of the feline c-myc exon 1 to be further downstream than the that given here. Since their report was based only upon Southern blot analysis using a murine exon 1 probe, and since the location I report has been supported by DNA sequencing and RNA S1 nuclease mapping (Chapter 5), the location Soe and Roy-Burman propose appears to be incorrect. This may be because the murine probe is less closely matched to feline c-myc exon 1 than is the human probe which I used (section 3.2.3).

3.3.3 Nucleotide sequence of the exon 1 region.

The sequencing strategy is shown in Fig.3.4. The sequence of the exon 1 region was of interest for several purposes:

(a) To confirm the location of exon 1.

(b) To compare its sequence to sequences of unknown origin at the 5' end of transduced FeLV v-myc genes.

(c) To investigate the function of this region of the gene. For example, could transcriptional control signals be identified?

(d) To facilitate analysis of c-myc transcripts in normal and tumour cells (Chapter 5).

3.3.4 Consensus sequences in the exon 1 region.

The sequence is presented in Fig.3.4. The putative 575 bp exon is enclosed within a box. The 5' limits were assigned to consensus RNA cap sites at positions 551 (P1) and 732 (P2) which were respectively 24 and 27 bp downstream from TATA boxes (Fig.3.5B). The P1 and P2 putative cap sites were homologous to those in the human and murine c-myc genes (Bernard et al, 1983; Watt et al, 1983b).

The 3' limit was identified by alignment with a consensus splice donor sequence, and was homologous with the splice donor identified in human and murine c-myc cDNA clones (Fig.3.5A; Stanton et al, 1984). That this site was a splice donor was supported by comparison with the structure of FeLV v-myc genes, which appear to be effectively spliced cDNA versions of the c-myc gene (Fig.3.5A).

Several putative binding sites for the SP1 transcription factor are also shown. Only sites which perfectly match the consensus sequence or its complement are shown (Dynan & Tjian, 1985). Several sites are clustered around the P1 and P2 region, in accord with their possible role in transcriptional initiation. It is noteworthy that the three sites immediately 5' to the P1 promoter-like region are identical in location and orientation to sites identified in the human c-myc gene from sequence and DNA footprinting studies (B. Whitelaw, J. Lang and N. Wilkie, pers. comm.).

3.3.5 Comparison of the c-myc exon 1 region in different species.

The sequence of exon 1 of the feline c-myc gene is 66% and 78% related to that of the mouse and man respectively. Exon 1 of the chicken c-myc gene bears no significant homology to that of the mammalian gene and is closer to exon 2 than is exon 1 of the cat, man and mouse (Shih et al, 1984; Linial & Groudine, 1985; Nottenburg & Varmus, 1986). The closest homology is between the cat and man, consistent with exons 2 and 3 also being most closely related between these species (Stewart et al, 1986b). The closer relationship between the feline and human genes at the DNA level follows the same tendency as overall genetic linkage where the feline and human linkage maps are more similar than are those of man and the mouse (O'Brien, 1986).

The 5' flanking sequences of the feline, human and murine c-myc genes are also highly related. This remarkable cross-species homology in a presumably non-coding region supports the proposal that this region performs a common control function (Siebenlist et al, 1984). Studies on the murine and human c-myc genes have identified a negative control element 0.4- 1.0 kb upstream of exon 1 (Remmers et al, 1986; B. Whitelaw et al, pers. comm.). The 550 bp region upstream of the feline c-myc gene is 78% homologous with the human gene and 68% with the mouse gene. The 130 bp immediately upstream of P1 including the three SP1 sites mentioned above, is 84% related to the human sequence. Most

mismatches are scattered nucleotide substitutions with only a few more extensive deletions or insertions. These sequence data suggest functional studies on the feline exon 1 region would reveal similar features as have been shown for the human and murine genes.

3.3.6 The coding potential of exon 1.

Exon 1 of the feline c-myc gene appears to be non-coding since it contains no translation initiation codons and stop codons are present in each reading frame, which agrees with reports on the human and murine c-myc genes (Fig.3.6; Bernard et al, 1983; Saito et al, 1983). Gazin et al (1983) reported the presence of a 188 codon open reading frame in the exon 1 region of the human gene. Although a homologous translation initiation codon is present at the P1 TATA box of the feline gene (reading frame 2, Fig.3.6), the putative product would be terminated after only 59 codons, and is not related to that reported for the human gene, except for 10/12 N-terminal residues. Related coding sequences are present further downstream, but these cross between the three reading frames. This divergence suggests that if exon 1 of the mammalian gene does code for a protein, it is not conserved between these species. This lack of conservation contrasts with the highly related reading frame of exons 2 and 3 of the human, mouse and feline genes (Bernard et al, 1983; Watt et al, 1983a; Stewart et al, 1986b).

Although it appears unlikely that feline c-myc exon 1 codes for a protein, this is not excluded if c-myc transcription could initiate further upstream than the presumptive P1 start site such that a translation initiation codon could be included in the mRNA (Fig.3.6). Bentley and Groudine (1986b) have recently reported the cloning of human c-myc cDNAs with multiple initiation sites originating from a promoter-like region (P0) ~500 bp upstream of P1. Also, Gazin et al (1986), have reported serological detection of a human cellular protein possibly related to the exon 1 product they had predicted earlier (Gazin et al, 1983).

3.3.7 Structural comparison of feline c-myc and v-myc genes.

Determination of the structure of the feline c-myc gene allowed comparison with FeLV v-myc genes to identify possible coding alterations. Fig.3.7 shows the structure of three independently isolated myc-transducing FeLVs. The LC, CT4 and FTT isolates each

contain the c-myc coding exons 2 and 3, which are invariably intact, with the qualified exception of FTT where a nonsense mutation terminates the v-myc reading frame two codons before the normal c-myc stop codon. Similarly, avian myc-transducing viruses (Reddy et al, 1983; Hayflick et al, 1985; Walther et al, 1986) contain intact myc N and C termini. This supports the view that the entire myc coding sequence without N- or C-terminal truncation is required for transforming activity, as indicated by recent mutagenesis studies on the MC29 v-myc gene (Heaney et al, 1986).

The FeLV v-myc genes shown contain portions of exon 1 (Fig.3.5). In contrast, avian v-myc genes do not, but instead contain sequences which may derive from intron 1. Assuming that the process of transduction begins with proviral integration 5' to the region to be transduced (Swanstrom et al, 1983), then this contrast between feline and chicken v-myc genes may reflect differences in the initial insertion sites at the c-myc genes in these species. This is supported by finding that ALV proviruses are integrated predominantly in intron 1 in chicken bursal lymphomas (Shih et al, 1984; Robinson & Gagnon, 1986), whereas FeLV proviruses are most commonly found upstream of exon 1 in feline thymic lymphomas (Chapter 4).

3.3.8 Predicted products of feline v-myc genes.

The v-myc product of the CT4 and FTT proviruses may be encoded by a spliced, sub-genomic RNA and translated from the normal c-myc initiation codon without fusion to any virus-coded products, since the v-myc genes are 3' to the the FeLV env gene splice acceptor signal (Fig.3.7). In contrast, FeLV-LC may express a gag-myc fusion product since its v-myc gene is joined in-frame with the gag p30 coding region. This fusion product would include 9 novel amino acids: five coded by exon 1 sequences and four by the short exon 2 leader.

Avian v-myc genes display similar modes of expression (Bister, 1984). Like FeLV-CT4 and -FTT, the MH2 and OK10 viruses may express their v-myc products from spliced sub-genomic RNAs. However, in contrast to FeLV, the location of the avian viral splice donor for producing sub-genomic env RNAs predicts that the myc products would be fused to the six N-terminal gag residues (Ficht et al, 1984). Similarly to FeLV-LC, expression of the v-myc genes of MC29 and CMII, and an alternative mode of expression of OK10 to that mentioned above,

would generate myc gene products which are fused to substantial viral structural gene components.

3.3.9 Coding differences between c-myc and v-myc genes.

The coding differences between feline c-myc and v-myc genes are listed in Table 3.2. FeLV-LC v-myc has no coding changes relative to c-myc, and the only predicted difference to normal c-myc is fusion to the gag component. This is strikingly similar to the avian CMII gag-myc product which is unchanged compared to c-myc except for a single amino acid substitution at the C-terminus (Walther et al, 1986).

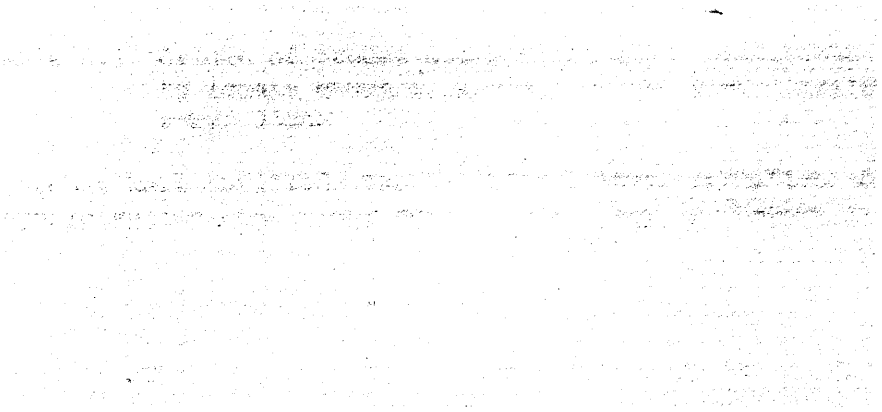
The FeLV-CT4 and -FTT v-myc genes show few coding changes relative to c-myc, and the amino acid substitutions present result from single nucleotide changes. FTT has one change at position 236 (Glu-Lys) and one which prematurely terminates the v-myc reading frame two codons from the end of the normal c-myc reading frame. CT4 has substitutions at positions 137 (Lys-Glu) and 214 (Ala-Thr) and a two residue insertion at position 438-439 due to a six nucleotide duplication.

There is no consistently altered codon in the three feline or four avian v-myc genes indicating that no specific amino acid change is obligately required for activation of the myc gene. The evidence from the LC and CMII v-myc genes indicates that no coding changes may be necessary. This view is supported by the sequences of several rearranged c-myc genes which are not altered compared to the normal gene (Battey et al, 1983; Wiman et al, 1984; Stanton et al, 1984).

Although specific codons are not consistently altered, there may be more general domains in which mutations would enhance myc oncogenic function. Such domains may be indicated as highly related regions of different members of the myc gene family. One particular domain, corresponding to residues 47-65 of the feline c-myc gene product, is identical in the c-myc genes of the cat, chicken, mouse, fish and man, and in the N-myc genes of the mouse and man (Stewart et al, 1986b; van Beveren et al, 1985; Van Beneden et al, 1986; DePinho et al, 1986; Stanton et al, 1986). Sequence data shows that this domain may be more frequently mutated than other regions in transduced and rearranged myc genes (Papas & Lautenberger, 1985; Westaway et al, 1984; Rabbitts et al, 1984; Showe et al, 1985; Murphy et al, 1986).

It is possible that coding changes augment the transforming activity of transduced myc genes. The avian MC29 and MH2 viruses and constructed

murine viruses carrying MC29 and MH2 v-myc genes, generally transform a wider spectrum of cell types than do the FeLV/myc viruses (GT3 and FTT) which have been studied so far (Hayman, 1983; Morse et al, 1986; D. Onions et al, in press). Perhaps the location and/or greater number of coding changes in avian v-myc genes partly determines a wider transforming spectrum. This may partly explain the altered transforming spectrum of the avian HB1 virus, derived from a transformation-defective MC29 myc deletion mutant, which has recovered c-myc sequences (Enrietto et al, 1983; Smith et al, 1985). However, the role of coding changes in myc oncogenic function remains to be systematically studied for the v-myc or c-myc genes of any species.



	EXON 1	EXON 2	EXON 3
Clone	pMC.41 c-*myc*	pCT4P2 v-*myc*	pCT4P1 v-*myc*
Species	human	cat	cat
Insert	XhoI/PvuII445	PstI450	PstI/EcoRI400
Hybridisation conditions	formamide: 30% temp. °C : 39	50% 42	50% 42
Wash conditions	temp. °C : 50 2X SSC	60 0.1X SSC	60 0.1X SSC

Table 3.1. Origin of probes and hybridisation conditions used to locate exons 1, 2 and 3 on the pFMC-1 feline c-*myc* clone.

Probes were purified inserts. Except where specified above, hybridisation conditions were as described in Chapter 2.

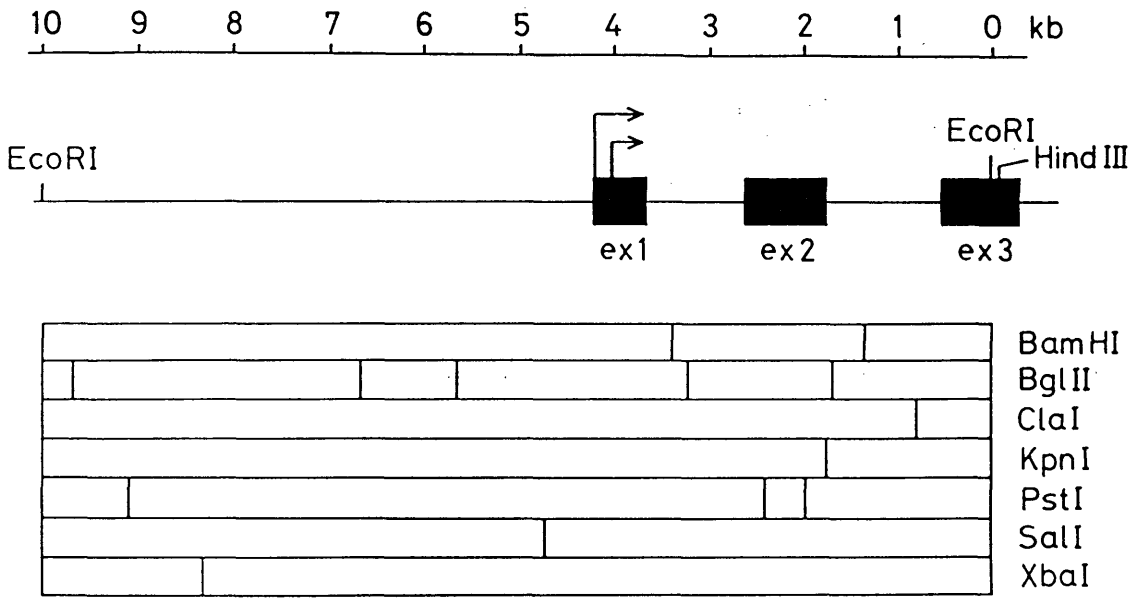


Fig.3.1. Structure of the feline c-myc gene.

Exons 1 to 3, indicated as black boxes are oriented 5' to 3' from left to right. The restriction enzyme map of the 10 kb EcoRI insert of the pFMC-1 clone is shown below.

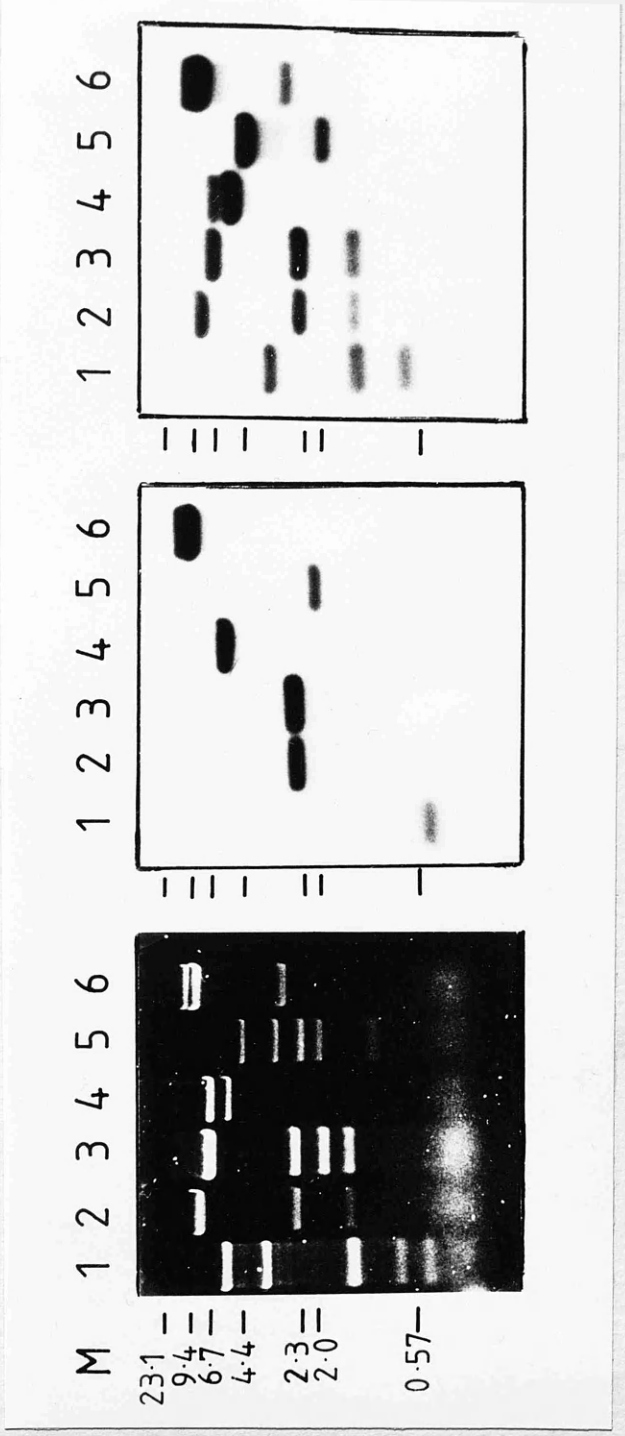


Fig.3.2. Location of c-myc exons 2 and 3 on clone pFMC-1.

An ethidium bromide stained gel of pFMC-1 digests is shown on the left. Duplicate Southern blots of this gel hybridised with exon 2 and 3-specific probes are shown in the centre and on the right respectively (Table 3.1). Lanes: 1, PstI/BamHI
2, BamHI
3, BamHI/SalI
4, SalI
5, BglII
6, EcoRI
M, HindIII-digested λDNA labelled with ^{32}P.

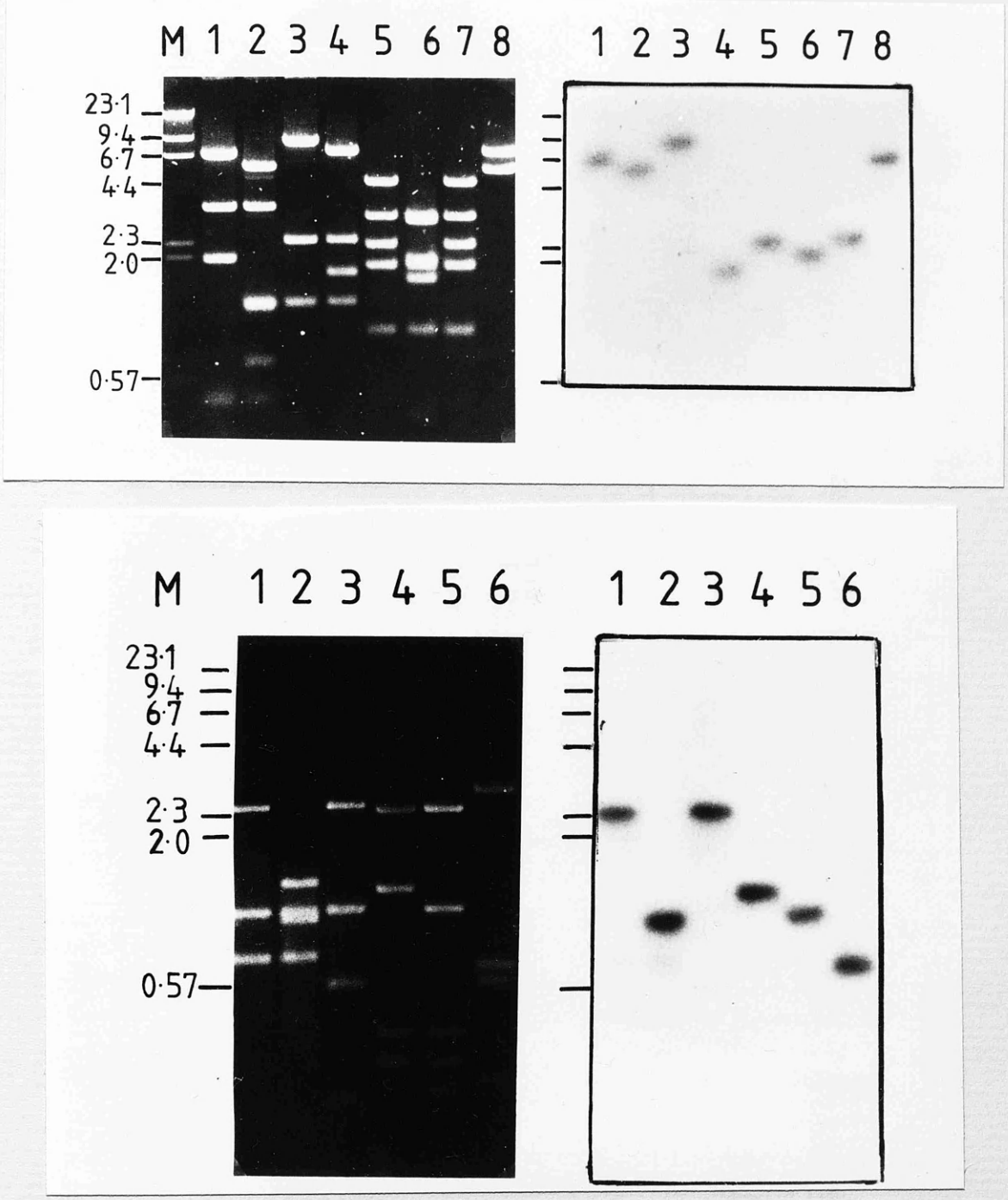
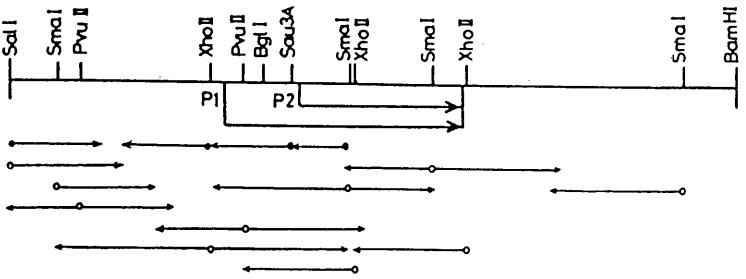


Fig.3.3. Location of exon 1 on clones (a) pFMC-1 (b) pSB1.8.

Ethidium bromide stained gels of plasmid digests are shown to the left of corresponding Southern blots hybridised with an exon 1-specific probe (Table 3.1). Lanes: M, HindIII-digested λDNA labelled with ^{32}P

(a) pFMC-1
1, PstI
2, PstI/BamHI
3, BamHI
4, BamHI/SalI
5, BglII
6, BglII/SalI
7, BglII/XbaI
8, XbaI/SalI

(b) pSB1.8
1, BglI
2, BglI/BamHI
3, BglI/SalI
4, PvuII
5, PvuII/BamHI
6, SmaI




```
   1 GTCGACACTAACACCCCACACCCTCGGAGCGCGCGCCCATAAATACCCCTTCTTCCCTTG   60
  61 TCTTCGCGGGATTTATTTTCCAAACGCTGCCCTTTCCCCAGCCTTCGGGAGGCGCCCGTC  120
 121 CACCCGGGACGTGCATGGCGTGGCGGTGGGTACACGGTGTGTTCCGTGTTGAAGGCTCAG  180
 181 CTGTTCCGCCTGCGATGATTTAAGCACGCAAGACAAGTATGCGGTTTGTCCAGCCGCGCG  240
 241 CTGCCGCGGAGGAGCACGGGAGAAAGGCAGCGGGTTTGAGCGGGAGCAAAAGAAAATGGT  300
 301 AGGCGCGCGCAGTTAATTCATGCTGCGCTCTGACTGTTTACATCCGATCGCTCGGCGCAC  360
 361 TGGAGTGCCAGGCAGAAAGGGCCGAGTCCTCCTCCCCAACCTCCCCCCCCCACCCCGCC  420
 421 CAGCCAGTCCTCCGCAGAAGCGCCCCTCCCCGGCTCCCAAAGCGGAGGGCGGGGGAGAGA  480
 481 GAAAAAAGATCCTCTCTCGCCGAATCCCCGCCCACCGGCCCTTTATAATGCGAGGGTCTGC  540
 541 GCGGTCGAGGACCCCCGAGCTGCGCTCTCCCCGCCGCAGCTGCCGCCGCCGGCTCGCGCC  600
 601 CCGGCTGCTGCCTGGCGCCCTGCCGGCTTCGGGAAGGGCAGGGCTATTCAGGGGCTTGGC  660
 661 GGGAAAAAGAGAACAGAGGGGAGGGATCGTGCCTAGCTCTGTATAAAAGCCGGTTCTTGG  720
 721 GGCTTTATCTGACTCTCTGTAGTAATCCTAGCAAGAGGCAGAGGGAGCGACGGGCGCGCC  780
 781 CGCCTGGGTAGAAGAGCAGAGCGAGGCGAGCGAGCCGAGTCCGGCTCCGGGCGCCCGGGG  840
 841 AAGGGAGATCCGGAGTGAAAGGGTGCTTCGCCTCCGGCCCACCCCACCCTGCCCGCCGCC  900
 901 CCCTGCCAGCGGTGCGCAACTCCCCGCCGCACCCGCGAAACTTTGCCGGTTGCGGCGGGC  960
 961 GGACACTGTTCCCTGGAACTTACAACACCCGAGCAAGAACGCGACTCTCCGGTCCGCCTA 1020
1021 TTTGGGGAAACACTTCTCCCCTACGCTGCCCGGGACCCGCTCCTCTGAAAGGGCGCTCCT 1080
1081 CGCCGCTTTTCGGACGCTGGATTTCCTTCGGATAGTGGAAAACCCGGTAAGCGCCCGGAT 1140
1141 CTATTTGTCTTCTCATTTATTTGCCCCACCGCTTGAATGCCGCGACGAGTCGAAGTCGAA 1200
1201 TGCCCCAATCGGGGGTCTTTTCTGCCATTCCTGCGCTATTGACACTTTTTCTCAGAGTAG 1260
1261 TTGTGGTAGCTCCGGGGTGGGGTGAGGACGACCCAGAACTGATGGGGTAGAGTGGCTTGT 1320
1321 CAGGATGGGAGGGCAAACCGGTGCGTTTTTGAAACTCGTCTTTAGAGATTTTGCTCATGT 1380
1381 ATACTGAGCGTCCCTGGACCGGTTGGACATTCCTTCGTTTATTCGATTAATTCGTTTTGA 1440
1441 TTTTTTGGAGCGGGGAGGGGGGGGGTGGGGAGGGAGTGCCGGCTTCGCGGTGGGCAGAGA 1500
1501 GCCCTCCCTGTACATCCCGAGCTCCTGGGAGTAAGAATTACAAATTGCTGTGGGAGCCGG 1560
1561 AGAGCGTCCGCCGCCGCTGACTTTTAGCGCTGGCTCAAAGGGACTTTAAATTTCGGCTTA 1620
1621 GCCACTTTGCTAGACGGCCTGTTGCTTCGCTGCGGCCTCGCCGCCCGGCATCCCCGGG   1678
```

Fig.3.4. Strategy and results of nucleotide sequence analysis of the exon 1 region of the feline c-myc gene.

Top: Strategy. A restriction map of the insert of plasmid pSB1.8 is shown with exon 1 in the centre. P1 and P2 indicate promoter-like elements. Arrows below the map show the direction in which fragments were sequenced. ⇢ indicates M13 clones, and ↔ fragments sequenced by chemical cleavage.

Bottom: Sequence. The exon boxed from positions 551-1126. Consensus sequences: P1 and P2 TATA boxes are marked (positions 535 and 712). Putative SP1 binding sites (GGGCGG or CCGCCC) are marked by small arrows under the sequence.

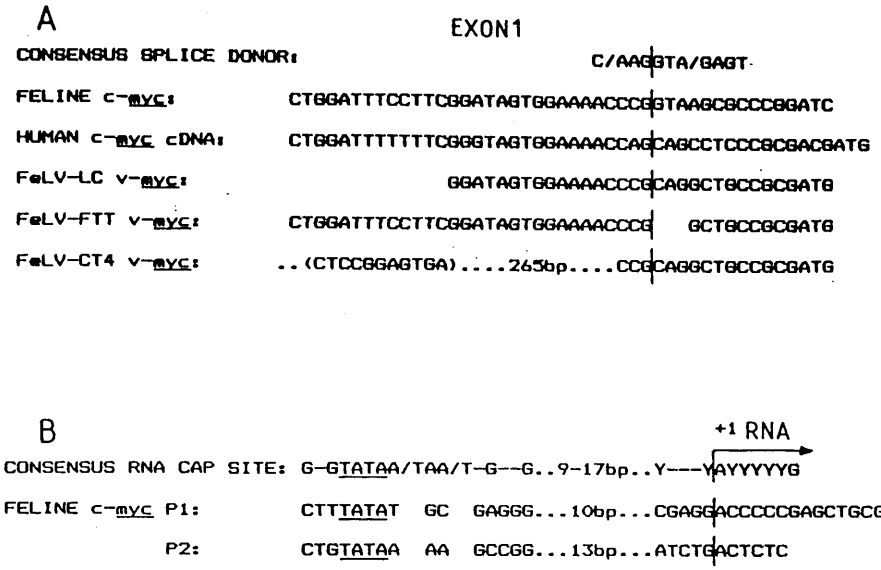


Fig.3.5. Alignment of sequences at the 5' and 3' ends of exon 1 with consensus promoter and splice donor signals.

(A) Splice donor. The sequence of the feline c-myc genomic clone is aligned with a consensus splice donor signal (Mount, 1982) and with the exon 1/exon 2 junction of a human c-myc cDNA clone (Watt et al, 1983a) and with the exon 1/exon 2 boundaries of clones of three FeLV v-myc genes (Braun et al, 1985; Stewart et al, 1986; D. Doggett et al, submitted). Exon 1 sequences present in these v-myc genes are shown in entirety.

(B) Promoters. The sequences of the feline c-myc P1 and P2 regions are aligned with a consensus TATA box and RNA cap site sequences (Breathnach & Chambon, 1981). (-) = any nucleotide; Y = pyrimidine.

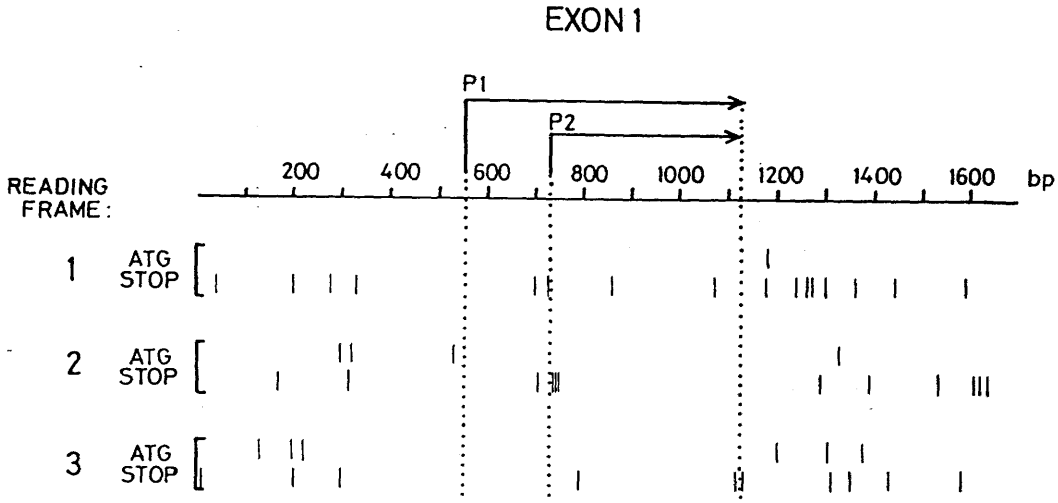


Fig.3.6. The coding potential of the exon 1 region of the feline c-myc gene.

The arrows marked P1 and P2 indicate the location of the exon within the sequenced region. Below this line the location of all potential translation initiation (ATG) and stop codons are shown as small vertical lines in each reading frame.

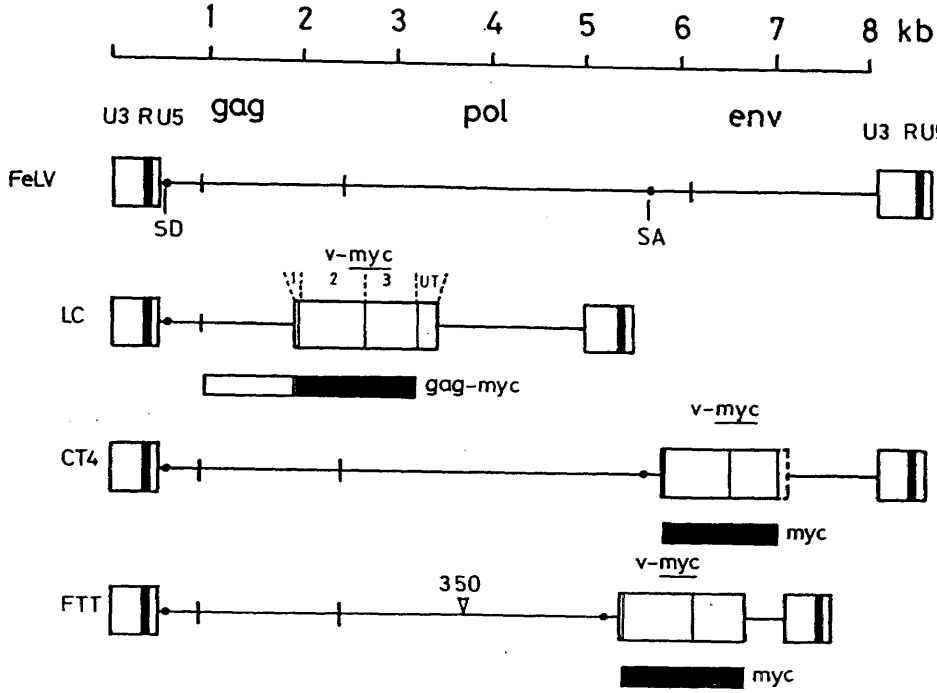


Fig.3.7. Structures of three FeLV/myc proviruses.

A non-recombinant FeLV provirus retaining gag, pol and env genes is shown for comparison. U3, R and U5 regions are marked in the 5' and 3' LTRs. SD and SA identify putative viral splice donor and acceptor sites for sub-genomic env transcripts. The v-myc structures were based on sequence data: LC, Braun et al, 1985; CT4, Stewart et al, 1986; FTT, D. Doggett et al, submitted.

Regions corresponding to c-myc exons 1, 2 and 3 are shown above the LC provirus; UT = c-myc exon 3 untranslated sequence. Predicted v-myc products are shown below each provirus as black boxes. The gag part of the LC gag-myc product is shown as an open box. A 350 bp deletion in pol is marked in the FTT provirus.

The CT4 clone 3' end was at the EcoRI site 3' to the c-myc translation stop codon; hence, the v-myc 3' terminus is marked as a dashed line to indicate this uncertainty. The presence of the CT4 3' LTR was shown by Southern blot analysis of the tumour DNA from which CT4 was isolated.

Normal c-myc gene		FeLV v-myc genes		
Codon	Amino acid	CT4	LC	FTT
157 AAA	Lys	GAA/Glu	-	-
214 GCC	Ala	ACC/Thr	-	-
236 GAG	Glu	-	-	AAG/Lys
285-286	-	TCTGCC/Ser-Ala insertion	-	-
359 CAC	His	-	-	GAC/Asp
438-439 TGTGCA	Cys-Ala	-	-	premature termination (TGAGCA)

Table 3.2. Coding differences between feline c-myc and v-myc genes.

Table compiled from sequence data in Braun et al, 1985; Stewart et al, 1986b; D. Doggett et al, submitted. (-) denotes no change in v-myc relative to c-myc sequence. In FTT v-myc, codon 438/TGT is changed to TGA/stop which terminates the reading frame two codons before the normal c-myc stop codon.

CHAPTER FOUR

REARRANGEMENTS OF THE c-myc GENE IN FELINE TUMOURS

4.1 INTRODUCTION

Rearrangements of the c-myc gene were first detected in ALV-induced chicken bursal lymphomas, where proviruses were inserted at the c-myc locus (Hayward et al, 1981). Then studies on human Burkitt's lymphomas showed that chromosomal translocations caused abnormal joining of immunoglobulin loci to the c-myc gene (Leder et al, 1983). This provided indirect evidence that the myc gene may be involved in oncogenesis by means other than transduction by avian retroviruses.

Since then, the c-myc gene has been found to be rearranged in a variety of tumours. Frequently one mode of rearrangement of the c-myc locus has been found to be characteristic in a given type of chicken, mouse or human tumour. Proviral insertions are commonly found in avian and murine retrovirus-induced bursal and thymic lymphomas respectively (Hayward et al, 1981; Robinson & Gagnon, 1986; Corcoran et al, 1984; Selten et al, 1984; O'Donnell et al, 1985), chromosomal translocations characterise some human and murine B-cell lymphomas (Leder et al, 1983; Marshall, 1985), and amplification has been found in various human carcinomas and a myeloid cell line (Alitalo et al, 1983; Collins & Groudine, 1982; Little et al, 1983).

FeLV-associated feline thymic lymphosarcomas provide another useful perspective concerning myc oncogenic potential since in these tumours, transduction of myc by FeLV and rearrangement of the c-myc locus have been observed (Neil et al, 1984; Levy et al, 1984; Mullins et al, 1984). Although study of myc transduction by FeLV has been facilitated by sequence analysis of three distinct v-myc genes and of the normal c-myc gene (Chapter 3) little information has been available regarding the nature of the feline c-myc gene rearrangements.

Preliminary Southern blot hybridisation data indicated that the c-myc gene was rearranged in several feline lymphoid tumours (Table 4.1). These include field cases and experimental tumours. All tumours were FeLV-positive thymic lymphosarcomas, except for case T9 which was an FeLV-negative spleen tumour. This chapter concerns characterisation of the rearrangements in more detail by hybridisation analysis of genomic DNAs.

4.2 RESULTS

To characterise the c-myc rearrangements, hybridisation analyses with c-myc-derived probes (shown in Fig.4.1) were performed on comparative restriction enzyme digests of tumour and control tissue DNA from the same animal. Restriction enzyme sites were located within the rearranged DNA relative to a reference site distal to the region of origin of the probe (Fig.4.5).

4.2.1 Proviral insertions in tumours T7, T8 and T24.

Preliminary analysis of tumour T24 DNA using probe 4 identified abnormal, tumour-specific HindIII and EcoRI bands which were <2 kb larger than the normal bands (Fig.4.2). This indicated the presence of a small insertion of <2 kb within the normal 10 kb EcoRI fragment since the EcoRI and HindIII fragments were increased in size to the same extent. This also showed that the insertion was between the EcoRI site 5' to exon 1 and the 3' most of two PstI sites in exon 2. The equal intensities of the normal and rearranged bands in tumour DNA suggested that the tumour consisted of a clonal population of cells carrying one mutant and one normal c-myc allele.

Further analysis using probe 1 on SalI/BamHI digested DNA detected an extra 3.0 kb fragment ~1.3 kb larger than the normal band, locating the insert within the SalI and BamHI sites which normally encompass exon 1 (Fig.4.3). That the insertion was upstream of the BamHI site in intron 1 was supported by digestion with SalI/BglII and SalI/HindIII which indicated that the BglII and HindIII sites had been displaced downstream by the same extent (~1.3 kb). SalI/PstI digestion revealed a normal 3.1 kb band and a 1.9 kb band suggesting that the insert introduced a new PstI site 1.9 kb 3' to the SalI site.

Probe 1 also detected an unexpected 4.5 kb band in some control DNA lanes (SalI/BglII, SalI/PstI) which is probably due to a contaminating plasmid, since its size does not agree with the normal c-myc map, and it is only present in some control and not tumour DNA lanes. For these reasons I have disregarded this band in discussing the results above.

Hybridisation of probe 2 to PstI/BamHI and PstI/BglII digested DNAs revealed no tumour-specific bands, confirming that the insert was 5' to the intron 1 BamHI site (Fig.4.3). Digestion with PstI and PstI/KpnI localised tumour-specific PstI and KpnI sites 2.4 and 2.1 kb

respectively upstream of the PstI site at the 5' end of exon 2. This suggested that the insert consisted of a truncated FeLV provirus retaining a 5' LTR orientated in the same transcriptional direction as c-myc, since PstI and KpnI sites are characteristic of the LTRs of exogenous FeLV proviruses (Mullins et al, 1980; Neil et al, 1984).

Using a similar approach the T7 and T8 rearrangements were shown to consist of approximately full-length FeLV proviruses retaining two LTRs, integrated 0.5 and 2.5 kb respectively upstream of c-myc exon 1, but in the opposite transcriptional direction to the gene (Fig.4.5). In tumour T8 it had previously been shown that FeLV U3 sequences were linked to the altered c-myc fragments (Neil et al, 1984). Again, these tumours retained a single normal allele at approximately the same intensity as the mutant allele (D. Forrest et al, submitted).

4.2.2 Rearrangement and amplification 3' to c-myc in tumour T5.

Tumour T5 presented a complex rearrangement at the 3' end of the c-myc gene. Analysis with probe 3 showed that the tumour-specific restriction enzyme sites were not typical of an FeLV provirus (Fig.4.4). Neither did analysis using a U3 probe specific for exogenous FeLV proviruses indicate linkage of the rearranged fragments and FeLV sequences (J. Neil, pers. comm.). Probe 3 detected no rearrangement upstream of the PstI site 0.5 kb 3' to the most 3' of the two exon 3 polyadenylation signals. The intensity of the signal was ~1.5 times that of the equivalent band in control DNA. On HindIII digested DNA, two tumour-specific bands of 4.3 and 4.0 kb and a normal band of 3.5 kb were detected, each of which was of approximately equal intensity. Upon HindIII/BamHI digestion the 4.3 kb band was the only rearranged fragment detected and this was at half the intensity of the normal 2.6 kb band. Similarly XbaI digestion revealed only one rearranged fragment (result not shown) but KpnI and SalI which map sites further downstream than the HindIII site 3.0 kb 3' to c-myc detected two tumour-specific bands.

These results indicated the presence of three c-myc alleles in tumour T5: two rearranged and one of normal gross structure (Fig.4.5). One alteration was between the PstI and BamHI sites 0.5 and 2.1 kb 3' to exon 3, and the other was between the XbaI and HindIII sites 2.5 and 3.0 kb 3' to exon 3. Since only one additional XbaI fragment was present, one of the mutant alleles appeared to retain the normal XbaI

site 2.5 kb 3' to exon 3. This indicated that the tumour-specific XbaI site was on the mutant c-myc allele which was rearranged 5' to the normal XbaI site, corresponding to the allele with the largest HindIII fragment. Further clarification of these structures would require cloning of the rearranged DNA.

4.3 DISCUSSION

Analysis of c-myc rearrangements revealed a variety of modes of alteration at the c-myc locus (Table 4.1; Fig.4.5). These include cases with a small degree of amplification (T5, T9), others due to proviral insertions (T7, T8, T24), and a case with a 3' rearrangement (T5). All rearrangements studied left the c-myc coding exons 2 and 3 intact, consistent with the suggestion made with respect to v-myc genes, that the entire myc product is required for transforming activity (Chapter 3). Similarly, no known c-myc rearrangement in human or murine tumours truncates the coding domain (Marshall, 1985).

4.3.1 Insertion in intron 1 in tumour T24

The insertion in intron 1 in tumour T24 appears to be a truncated FeLV provirus retaining a 5' LTR in the same orientation as c-myc and is reminiscent of ALV and REV promoter insertions at c-myc in chicken bursal lymphomas, although in these cases it is usually a 3'LTR which is retained (Hayward et al, 1981; Shih et al, 1984; Swift et al, 1985). Viral promoter insertion may be favoured by integration in intron 1 since this displaces the normal cellular gene promoter region.

More than 70% of ALV and REV insertions in the chicken c-myc locus are in intron 1 (Shih et al, 1984; Swift et al, 1985; Robinson & Gagnon, 1986) which may signify that this integration site favours activation of the gene, perhaps by displacing exon 1 and upstream regulatory sequences (Saito et al, 1983; Piechaczyk et al, 1985; Bentley & Groudine, 1986a). In contrast, most insertions at the feline or murine c-myc gene are upstream of exon 1 (Fig.4.5; J. Mullins, pers. comm.; Corcoran et al, 1984; Selten et al, 1984; O'Donnell et al, 1985). It is possible that different regions are locally susceptible to proviral insertion in these species, perhaps due to an open chromatin structure (Shih et al, 1984; Robinson & Gagnon, 1986). This is not to argue that they are fortuitous common integration sites irrelevant to oncogenesis, since some studies show

ALV, MuLV and FeLV-infected, non-transformed tissues not to display specific integration patterns (Fung et al, 1982; van der Putten et al, 1979; Casey et al, 1981).

Many avian proviruses at the c-myc locus, like the T24 insert, are deleted to some extent (Fung et al, 1981; Swift et al, 1985; Robinson & Gagnon, 1986). Most commonly, internal sequences near to the 5' LTR are deleted, perhaps including a splice donor site and viral RNA packaging signals (Robinson & Gagnon, 1986). Since some tumours contain only a deleted provirus, it was suggested that deletions may be selected if they prevent expression of viral antigens, allowing the tumour to escape the host's immune response (Payne et al, 1981; Neel et al, 1981).

4.3.2 Upstream insertions in tumours T7 and T8

The apparently intact FeLV proviruses upstream of c-myc in tumours T7 and T8 are oriented away from the gene, similar to the enhancer insertions found in many MuLV-induced thymic lymphomas and some ALV-induced bursal lymphomas (Corcoran et al, 1984; Selten et al, 1984; Li et al, 1984; Steffen, 1984; Payne et al, 1982). Why are upstream proviruses predominantly in this orientation? An explanation proposed for similar findings on MMTV insertions at int-1 and int-2 is that since enhancers may act preferentially on proximal promoters, then this orientation may be required to juxtapose the 5' LTR enhancer to the cellular gene promoters (Nusse et al, 1984; Dickson et al, 1984). If the provirus was in the other orientation then a viral promoter would lie between the enhancer in the 3' LTR and the cellular gene promoter.

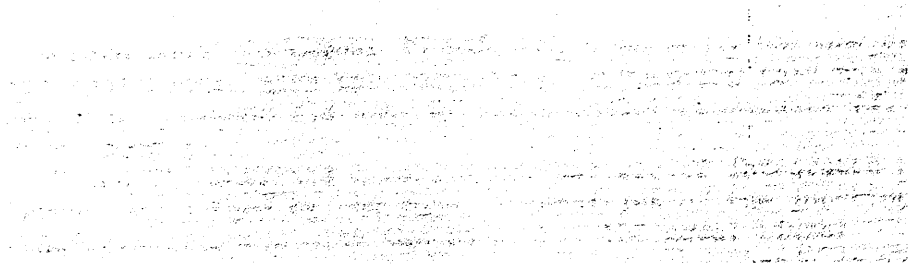
Upstream insertions may superimpose viral LTR control over the c-myc promoters, but may also disrupt regulatory elements identified upstream of the mammalian c-myc gene (Remmers et al, 1986; B. Whitelaw et al, pers. comm.) as has been proposed in murine plasmacytomas where the c-myc gene carries an upstream chromosomal translocation breakpoint (Yang et al, 1985).

4.3.3 Alteration at the 3' end of c-myc in tumour T5

Tumour T5 presents a complex 3' rearrangement which is atypical of those recorded in retrovirus-associated lymphomas of other species, since it does not seem to be directly virus-induced. The events generating the two rearranged alleles are not known but must involve

duplication and two structural alterations. This structure is remarkably like that found in the human leukaemic T cell line Hut78 which carries two copies of a mutant c-myc allele rearranged 0.6-1.0 kb 3' of exon 3 (Saglio et al, 1986). In this case the two mutant alleles appear to result from duplication of the entire chromosome. Unfortunately, tumour T5 could not be analysed cytogenetically as the cells could not be established in culture (D. Onions, pers. comm.).

Although the origin of the rearranged DNA 3' to c-myc is unknown, recent studies of human T cell leukaemias suggest possibilities for further study. Chromosomal translocations have been identified which abnormally join the α-chain locus of the T cell antigen receptor (TCR) 3' to c-myc (Erikson et al, 1986; Shima et al, 1986). These rearrangements are similar to variant translocations in Burkitt's lymphomas which join Ig light chain genes 3' to c-myc (Taub et al, 1984b; Hollis et al, 1984). 3' rearrangements may deregulate c-myc expression through a long range mechanism, perhaps involving TCR or Ig gene enhancers which may be highly active in T or B cells respectively. Although no linkage between exogenous FeLV sequences and the T5 rearranged c-myc fragments was detected, a few FeLV proviral elements were present (J. Neil, pers. comm.). It is an intriguing possibility that chromosomal rearrangement at c-myc and an independent FeLV insertion at another cellular locus were involved in the genesis of the tumour.



TUMOUR CASE	FIELD OR EXPERIMENTAL CASE	FeLV STATUS	FeLV COPY NO.	FeLV STRAIN	DISEASE LATENCY (months)	c-myc ALTERATION
T9	field	-	0	/	/	amplification
T7	"	+	11	/	/	5' insertion
T8	experimental	+	>50	Rickard	6	5' insertion
T5	"	+	(2)	Glasgow-1	24	3' alteration + amplification
T24	"	+	ND	Glasgow-1	9	5' insertion

Table 4.1. Characteristics of feline tumours which contain a rearranged c-myc gene.

(/) = not applicable.
ND = not determined.

FeLV status refers to the presence of integrated exogenous FeLV proviruses detected by a U3 hybridisation probe. The copy number of proviruses was assessed by the number of discrete FeLV-cellular DNA junction fragments detected with the U3 probe on KpnI digested tumour DNA (KpnI cuts in the R region of the LTR of a typical FeLV provirus). This analysis detected two discrete bands in tumour T5 DNA, but apparently not any proviral internal bands, with KpnI digestion, suggesting that the FeLV sequences present did not represent intact proviruses (J. Neil, pers. comm.).

T7 and T5 contained a monoclonal pattern of integrated FeLV sequences, judged by the equal intensities of the junction fragments detected with the U3 probe. The equal signal intensities of the normal and rearranged c-myc bands in T7, T8 and T24 indicated that these were clonal tumours (section 4.2.1). Clonality could not be assessed for T9 using these probes.

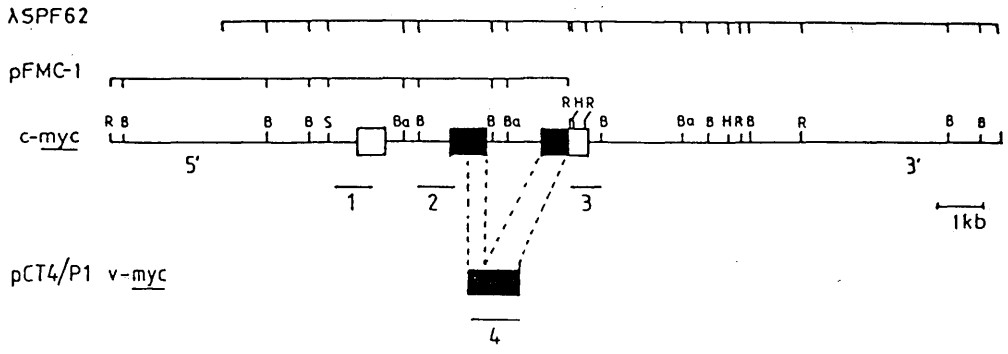


Fig.4.1. Origin of probes used to map c-myc rearrangements in feline tumours.

The top two lines represent overlapping clones of the feline c-myc gene (λSPF62, Mullins et al, 1984; pFMC-1, Chapter 3). The next line shows a restriction enzyme map of the c-myc locus with exons 1-3 represented as boxes from left to right.

Probes: 1, SmaI/700 bp; 2, BglII/PstI/500 bp; 3, HindIII/BglII/645 bp; 4, PstI/EcoRI/700 bp derived from pCT4 v-myc clone (Neil et al, 1984). Restriction enzyme abbreviations: Ba, BamHI; B, BglII, H, HindIII; R, EcoRI; S, SalI.

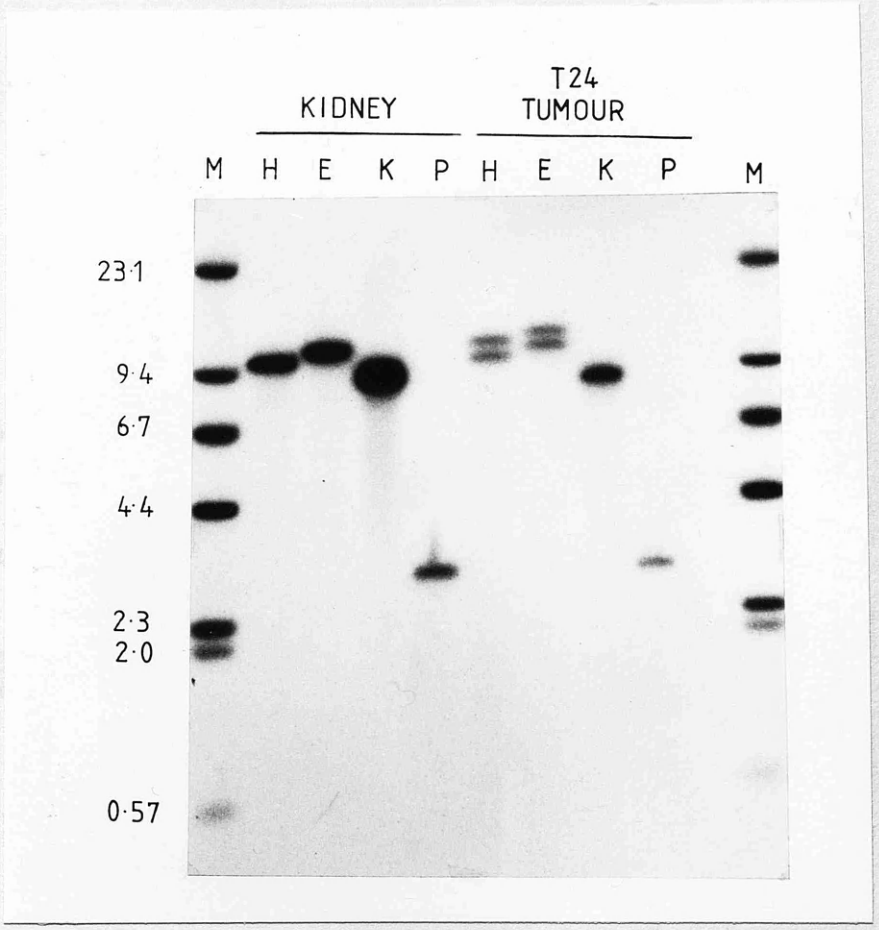


Fig.4.2. Southern blot analysis of tumour T24 and control kidney DNA using probe 4 (Fig.4.1).

The different signal intensities between tumour and control lanes result from variations in DNA concentrations.
Restriction enzyme abbreviations: H, HindIII; E, EcoRI; K, KpnI; P, PstI.
M, HindIII-digested λDNA, ^{32}P-labelled markers.

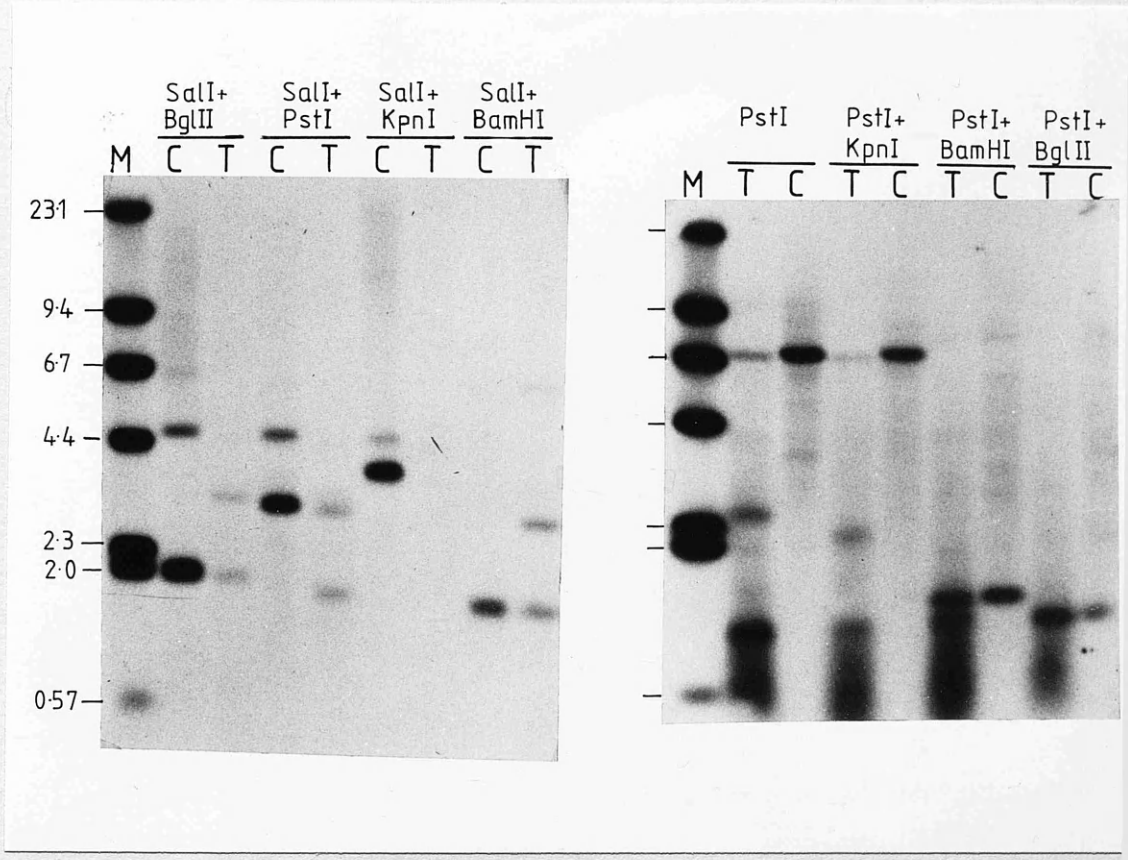


Fig.4.3. Southern blot analysis of control kidney (C) and tumour T24 DNA using probe 1 (left) and probe 2 (right) (Fig.4.1).

M, HindIII-digested λDNA, ^{32}P-labelled markers.
Probe 2 gave a background signal in all lanes which may be due to a moderately repetitive element in the intron 1 region from which probe 2 originated. The stronger smear in the <1 kb range in tumour lanes may be due to hybridisation to PstI satellite bands and to RNA present in the DNA preparation.

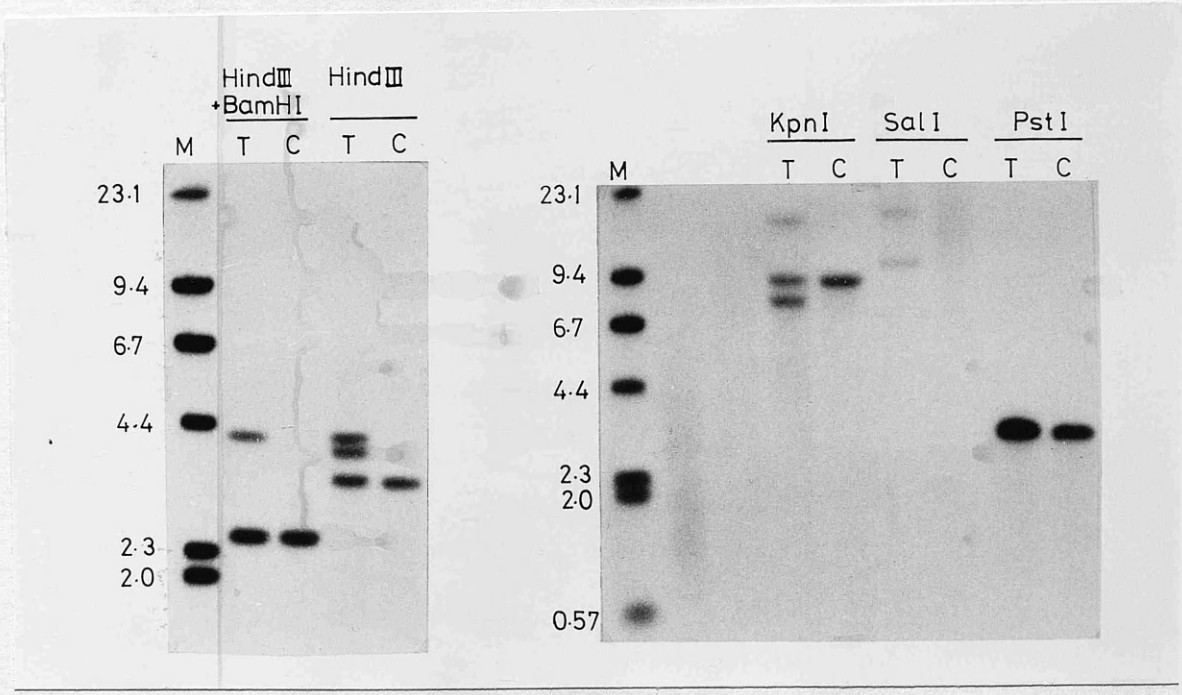


Fig.4.4. Southern blot analysis of control kidney (C) and Tumour T5 (T) DNA using probe 3 (Fig.4.1).

M, HindIII-digested λDNA ^{32}P-labelled markers.
The reduced signal in the high molecular weight range results from poorer transfer of large DNA fragments.

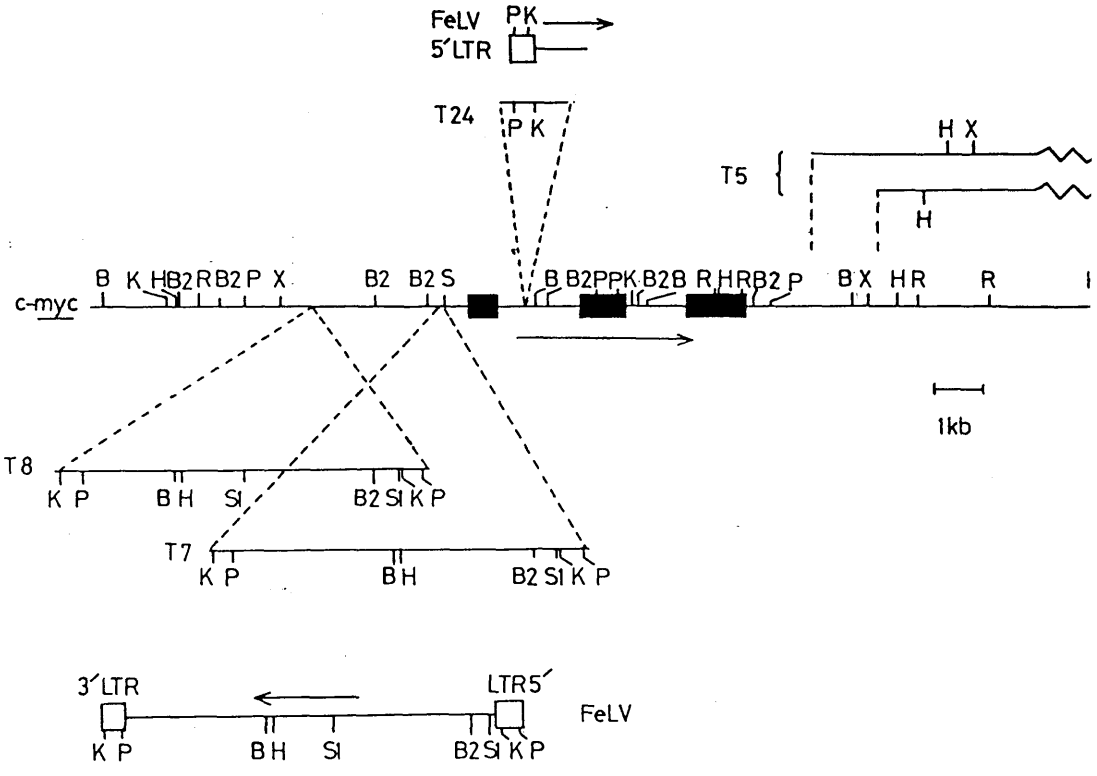


Fig.4.5. Structures of rearranged c-myc genes in tumours.

The central line represents a restriction enzyme map of the normal c-myc locus with exons 1 - 3 shown as black boxes, from left to right. To aid interpretation, a typical restriction enzyme map of an FeLV provirus and that of a truncated provirus retaining a 5' LTR are shown at the top and bottom of the diagram for comparison to the rearranged structures. Relative transcriptional orientations of c-myc and FeLV sequences are shown by horizontal arrows.

Restriction enzyme abbreviations: B, BamHI; B2, BglII; H, HindIII; K, KpnI; P, PstI; R, EcoRI; S, SalI; S1, SstI; X, XbaI.

CHAPTER FIVE

EXPRESSION OF THE c-myc GENE IN NORMAL AND TUMOUR CELLS

5.1 INTRODUCTION

It was necessary to study the structure and expression of the transcription unit of the normal feline c-myc gene, so that comparison could be made with tumour cases containing rearranged c-myc loci. This could provide clues as to how expression may be altered possibly causing oncogenic activation of the gene in these tumours.

Previous reports showed that the human and murine c-myc genes produce transcripts from two promoter regions, P1 and P2, separated by ~150 bp at the 5' end of exon 1 (Watt et al, 1983b; Stewart et al, 1984a). The sequence of the feline c-myc gene identified similar P1 and P2 regions, hence it was of interest to investigate the presence of corresponding transcripts. Also, two possible polyadenylation sites had been identified at the 3' end of exon 3 in the human, mouse, chicken and cat genes, but investigation of their function had previously been reported only for the human gene (ar-Rushdi et al, 1983; Taub et al, 1984b).

This chapter also includes an examination of how expression of the feline c-myc gene might be altered by rearrangements in tumours. Some experiments were prompted by reports on altered c-myc genes in tumours of other species. First, it was reported that rearrangement of the c-myc gene may elevate its expression (Hayward et al, 1981; Alitalo et al, 1983; Erikson et al, 1983). This has been interpreted as evidence that c-myc expression has become deregulated in a way that contributes to oncogenesis. Also, it has been proposed that in human and mouse B cell lymphomas, rearrangements of the c-myc gene which leave the transcription unit intact may disturb the normal balance in relative abundance of the P1 and P2 RNAs (Taub et al, 1984a; Yang et al, 1985). An elevated ratio of P1 to P2 RNAs may be characteristic of these rearrangements, and has been suggested to reflect oncogenic activation of the gene, perhaps under the control of cis-acting elements present in the rearranged DNA and/or by disruption of control regions of the c-myc locus (Remmers et al, 1986). In contrast, in tumours where rearrangements disrupt exon 1 of the c-myc gene it has been suggested that altered c-myc RNAs are produced which have abnormal

properties, perhaps through loss of exon 1 regulatory functions (Saito et al, 1983; Piechaczyk et al, 1985).

Analysis of the limits of the transcription unit of the feline c-myc gene involved S1 nuclease mapping of RNA from normal and tumour cells with probes derived from appropriate regions of clones of the normal gene, as predicted from sequence data.

5.2 MATERIALS AND METHODS

All procedures were as described previously except the following:

5.2.1 Materials.

DMSO/dye: 30% (w/v) dimethylsulphoxide, 1mM EDTA, 0.05% (w/v) of each of xylene cyanol and bromophenol blue (stored at 4°C).

S1 nuclease mapping hybridisation solution: 80% (v/v) formamide, 40mM PIPES buffer pH 6.4, 400mM NaCl, 1 mM EDTA (stored at -20°C).

S1 nuclease digestion solution: 200mM NaCl, 4.5mM zinc acetate, 80mM sodium acetate pH4.4, 20µg/ml calf thymus DNA (stored at -20°C).

Glass microcapillary tubes were coated internally before use with repelcote, and were treated with DEP to inactivate possible contaminating RNAses.

5.2.2 Methods.

Preparation of 5' and 3' end-labelled, single-stranded probes.

Fig.5.1 indicates the c-myc-derived S1 nuclease mapping probes used and Table 5.1 summarises the protocols for their preparation and use.

Probe A. The SmaI 700 bp fragment was sub-cloned into pUC8 to generate the plasmid pS700. 10µg aliquots of pS700 were digested with SmaI then treated with bacterial alkaline phosphatase, and 5' end-labelled with polynucleotide kinase and γ-^{32}P-ATP (Chapter 2). Unincorporated nucleotides were removed by gel filtration through a Sephadex G50 column. The labelled SmaI 700 bp insert was purified by preparative agarose gel electrophoresis.

Strand-separation: the purified fragment was dissolved in denaturing DMSO/dye mixture, heated at 90°C for 4 minutes to dissociate the DNA strands, then the tube was plunged immediately into iced water according to Maniatis et al (1982). The mixture was loaded onto a 5% polyacrylamide strand-separating gel (40 x 20 cm x 1.5 mm) and electrophoresed for 18-20h at 300V. The separated strands were located on the gel by autoradiography (Fig.5.2). Both strands were eluted from

the gel by standard methods (Chapter 2). It was empirically determined by S1 nuclease analyses that the upper band contained the antisense strand (i.e. complementary to the RNA).

Probe B was prepared by essentially the same method as for probe A, except that strand-separation was performed on an 8% polyacrylamide gel at 200V for 18h. The upper band was determined to contain the antisense strand.

Probe C. The 3' ends of probe C (HindIII/BglII 650 bp) were labelled using Klenow polymerase (Chapter 2). 15µg of a plasmid (obtained from R. McFarlane) containing the probe C fragment, digested with HindIII and BglII, was dissolved in 15µl of TE pH8.0. This was made to a volume of 50µl containing 1mM DTT, 1mM spermidine, 1x Klenow reaction buffer, 10 units of Klenow polymerase and 60µCi of each of all four α-^{32}P-labelled dNTPs. The reaction was incubated at room temperature for 15 minutes, then 10µl of chase solution (0.5mM of each non-radioactive dNTP) added, and the reaction incubated for 20 minutes longer. Unincorporated nucleotides were removed and the labelled probe fragment isolated as described for probe A. The strands were separated on a 5% polyacrylamide gel by electrophoresis at 320V for 20h. The lower band contained the antisense strand.

S1 nuclease analyses.

20µg samples of total cellular RNA were added to a 10µl volume of hybridisation solution with 10µg carrier *E. coli* rRNA and 100ng of the relevant probe A, B or C. (A titration experiment with probe A determined that 100ng should ensure conditions of probe excess; section 5.3.1). The reactions were performed in sealed glass microcapillary tubes which were completely immersed in a water bath at 95°C for 5 minutes then immediately transferred into a water bath at the appropriate temperature for overnight incubation (Table 5.1). Next day the tubes were removed, carefully opened, and the contents ejected into 100µl of ice-cold S1 nuclease digestion buffer. 100 units of S1 nuclease were added and the reaction incubated at 37°C for 0.5h. Reactions were stopped by placing on ice and adding 4µl of 0.5M EDTA. Carrier yeast tRNA (20µg) was added and the mixture was phenol extracted and ethanol precipitated. Samples were redissolved in 4µl of formamide/dye, heated at 95°C for 5 minutes then electrophoresed on 6% (probes A and B) or 4% (probe C) polyacrylamide/7M urea gels (Chapter

2), at 1200-1500V. Gels were fixed in 10% (v/v) acetic acid/10% (v/v) methanol, dried, then autoradiographed at -70°C.

5.3 RESULTS

5.3.1 Determination of optimum hybridisation conditions. (Table 5.1).

Various factors influence the formation and stability of DNA/RNA hybrids in S1 nuclease analyses (Favoloro et al, 1980), including the size of the hybrid formed and the %GC base-pairing between the probe and RNA. Ideally, the hybridisation temperature should be as high as possible to minimise non-specific annealing and to reduce formation of intra-molecular secondary structures, but not so high that the DNA/RNA hybrids are unstable.

Probe A. It was important to determine the optimum hybridisation temperature and the conditions for probe excess for probe A, as this was to be used for quantitative analysis of the relative abundances of the P1 and P2 5' end discontinuities:

Temperature: RNA from tumour 86800 which has no detectable myc alteration, was hybridised with probe A over a range of temperatures (51-67°C) (Fig.5.3). Probe A detected P1 and P2 RNAs as S1 nuclease protected bands of 286 and 105 nucleotides. The ratio of P1 to P2 bands detected varied from 6:1 at 51°C to 3:1 at 63°C. At 67°C no P2 signal could be detected, even with prolonged autoradiography, suggesting that 67°C incubation destabilised the shorter P2 hybrids. I performed subsequent analyses at 59°C to optimise the P2 signal.

Probe concentration: Using probe A to analyse tumour 86800 RNA, the P1/P2 ratio detected was found to vary with the amount of probe (Fig.5.4). A constant ratio was detected with >50 ng of probe. In subsequent analyses, I used 100 ng of probe per reaction to ensure conditions of probe excess for other samples since none seemed to contain markedly higher levels of c-myc RNA than tumour 86800.

Probe B was used under the conditions determined for probe A, since it was expected to detect P1 hybrids of intermediate length and similar %GC base-pairing as the P1 and P2 bands detected with probe A.

Probe C was used to map RNA at the 3' end of exon 3. (Fig.5.5). Only one major band of ~435 nucleotides was detected over the chosen temperature range. Above 49°C no obvious bands were detected, suggesting that this temperature destabilised hybrids. I performed

later analyses at 41°C.

5.3.2 5' and 3' limits of the c-myc transcription unit (Fig.5.6).

Probe A detected P1 and P2 5' discontinuities of 286 and 105 nucleotides in RNA from normal feline embryonic fibroblasts and tumour 86800. Probe B was used as an independent confirmation for P1 hybrids which were detected as a series of bands of ~138 nucleotides (see section 5.3.3).

Probe C detected a major band (~435 nucleotides) in normal fibroblast RNA mapping ~25 nucleotides 3' to the most downstream polyadenylation signal (Fig.5.8). Additional fainter bands were detected below this major band, one of which (~310 nucleotides) mapped ~15 nucleotides 3' to the upstream polyadenylation signal. However, the strongest of these minor bands was only ~10 nucleotides shorter than the major band, and could result from excessive S1 nuclease digestion or stem-loop formation.

5.3.3 Mapping P1 and P2 RNA 5' discontinuities to the exon 1 sequence

P1 and P2 bands were precisely located against sequence ladders of probes A and B, and mapped to consensus RNA cap sites 24 and 27 nucleotides respectively downstream from TATA boxes (Fig.5.7). The broad P1 band mapped over three nucleotides at the expected RNA start site, and included a fainter band seven nucleotides shorter, present in all normal and tumour RNAs studied. This minor band may represent another RNA species with a different 5 ' terminus since it maps to a sequence with some homology to an RNA cap site. However, since it is so close to the major band detected, it may result from excessive S1 nuclease digestion or secondary structure formation. The broad P2 band mapped over four nucleotides at the predicted P2 consensus cap site.

5.3.4 Levels of expression of c-myc in normal and tumour cells.

Northern blot analyses with a myc probe of RNA from tumours with or without c-myc rearrangements, others containing an FeLV/myc virus or with no apparent myc alteration, and from normal cells are shown in Fig.5.9. Levels of c-myc RNA were not markedly elevated in tumours T7, T8, T5 and T24 with c-myc rearrangements compared to tumours with no myc abnormality. Levels were greater than in normal embryo fibroblasts and mitogen stimulated thymocytes, but did not show the dramatic increase observed for a myc gene expressed from a recombinant FeLV/myc provirus.

5.3.5 Normal c-myc RNA structure in tumours T7, T8 and T5.

Northern blot analysis detected c-myc RNA of normal size (2.2-2.4 kb) in tumours T7, T8 and T5, in accord with Southern blot mapping data showing that the rearrangements in these cases leave the normal transcription unit intact (Fig.5.9; Chapter 4). In view of the proximity of the T7, T8 and T5 rearrangements to the c-myc transcription unit, I undertook S1 nuclease mapping to investigate possible subtle changes to the termini of c-myc transcripts. Using probe A, P1 and P2 5' discontinuities were detected in cases T7, T8 and T5 and in tumours without c-myc rearrangement, which were co-terminal with those in normal fibroblasts and thymocytes.

Normal 3' ends were detected in all tumours studied regardless of c-myc rearrangement (Fig.5.10B). Thus, in tumour T5 the 3' alterations to c-myc on the two mutant alleles do not appear to perturb formation of the normal transcript 3' end.

5.3.6 Loss of exon 1 in tumour T24 c-myc transcripts.

Northern blot analysis showed that the c-myc RNA in tumour T24 was ~200 nucleotides shorter than normal (Fig.5.9), and S1 nuclease mapping detected no P1 and P2 bands, consistent with Southern blot data showing that the insertion displaced exon 1 (Chapter 4). Interestingly, lack of P1 and P2 bands indicated that there was no detectable RNA from the unrearranged c-myc allele in this tumour. Since Northern blot analysis detected a c-myc RNA band, and since probe C (Fig.5.10B) detected a normal RNA 3' end, this RNA was probably derived from the mutant, not the normal, allele (discussed in Chapter 6).

5.3.7 Varying ratios of P1 to P2 RNAs in tumours and normal cells.

The P1/P2 ratios in the tumour series varied considerably (Fig.5.9; Table 5.2). In normal fibroblasts and in alimentary lymphosarcoma 87416, approximately equal levels of P1 and P2 transcripts were detected. In ConA stimulated normal thymocytes and nine T cell tumours a higher ratio was detected. In normal thymocytes the ratio was ~2:1 but in tumours this varied from 3.1:1 (case 86800) to 19.6:1 (case 89407). Tumours T7, T8 and T5 displayed ratios in the highest range (17.9:1, 17.0:1 and 13.6:1 respectively). T cell tumours without detectable alteration in the 20 kb region around the c-myc locus screened by Southern blot analysis displayed a range of ratios , which in some cases (T21 and 89407) were comparable to those in tumours

carrying a c-myc rearrangement. No correlation was observed between the ratio and the presence or absence of FeLV.

The P1/P2 ratio in feline thymic tumours was markedly higher (in the range 3:1 to 20:1) than that recorded for human and mouse B cell lymphomas with or without c-myc rearrangements (generally < 2:1; Taub et al, 1984b; Yang et al,, 1985). The differences may result partly from different S1 nuclease analysis conditions, since my results in section 5.3.1 show the importance of standardising conditions. However, the differences may also reflect genuine species and/or cell type variations.

5.4 DISCUSSION

5.4.1 The transcription unit of the feline c-myc gene.

S1 nuclease mapping supports the predicted locations of signals for initiation and polyadenylation of transcripts of the feline c-myc gene. The P1 and P2 5' discontinuities mapped to promoter-like sequences, and although unequivocal confirmation that these represent RNA start sites and not splice acceptor sites would require primer extension studies, this view is supported by other evidence:

(1) The sequences around the P1 and P2 sites closely match the consensus for TATA boxes and RNA cap sites (Fig.5.7) but do not conform closely to splice acceptor signals (Mount, 1982).

(2) The P1 and P2 sites were homologous to sites identified in the human and mouse c-myc genes by S1 nuclease mapping (Watt et al, 1983b; Stewart et al, 1984a). The human P1 site has been confirmed by primer extension analysis (Watt et al, 1983b).

(3) The same P1 and P2 S1 nuclease protected bands were detected in two tumours with FeLV insertions 0.5 and 2.5 kb upstream of exon 1 (section 5.3.5) suggesting that the insertions lie outside the transcription unit.

S1 nuclease mapping of the 3' end of exon 3 suggested that the most 3' of the two possible polyadenylation signals is used predominantly as seems to be the case in the human and chicken c-myc genes (ar-Rushdi et al, 1983; Taub et al, 1984a; Nottenburg & Varmus, 1986). Other elements near the AATAAA signal also may be important to form the 3' end of eukaryotic transcripts. McLauchlan et al (1985) identified a consensus sequence located ~30 nucleotides 3' to the AATAAA site in 70%

of genes examined, and a closely matched sequence (AGTGTTTT) occurs 41 nucleotides 3' to the most 3' AATAAA signal of the feline c-myc gene (Fig.5.8).

5.4.2 Significance of the P1 and P2 c-myc transcripts.

No differential function is known for the P1 and P2 transcripts of the mammalian c-myc gene. The exon 1 leader of the RNA may adopt secondary structures, some of which may differ between the P1 and P2 RNAs (Battey et al, 1983), but their physiological relevance is unknown.

Since P1 and P2 RNAs are reported to be equally stable (Piechaczyk et al, 1985) variations in the P1/P2 ratio suggest that they may be differentially transcribed. My results suggest that the P1/P2 ratio may differ in a tissue-specific fashion, as the ratio in fibrobasts was less than half that in thymocytes (Table 5.2). Also, the ratio was considerably higher in T-cell tumours than in an alimentary lymphosarcoma (probably a B-cell tumour; Jarrett, 1984). Similarly, variations in the ratio in different mouse tissues have been recorded (Stewart et al, 1984a).

5.4.3 Rearranged c-myc genes: levels of expression.

Levels of c-myc RNA do not differ markedly in feline tumours with or without rearrangement of the gene, although tumour T5 carrying a small degree of amplification of the c-myc gene has slightly higher levels. However, levels are higher than in normal cells (fibroblasts or thymocytes) as also reported for chicken and mouse tumours with proviral insertions at the c-myc gene (Hayward et al, 1981; Payne et al, 1982; Corcoran et al, 1984; Selten et al, 1984).

It is difficult to assess the significance of the levels of c-myc RNA in the tumours with rearrangements of the gene since an appropriate control cell is subject to arbitrary choice. Neither normal cells nor tumours without an altered c-myc gene may be appropriate. Perhaps the ideal control would differ from the tumour cells with the c-myc rearrangement only in the structure of its c-myc gene. Since the tumours may arise from an infected cell at an unknown point in the T-cell developmental pathway, the precise control cell would not be available.

5.4.4 Structure of transcripts from rearranged c-myc genes

Of the feline tumours studied, only case T24 contained an unusual

c-myc RNA, ~200 nucleotides shorter than normal, apparently resulting from displacement of exon 1. Loss of exon 1 in rearranged c-myc genes in human and mouse tumours has been reported to increase c-myc RNA levels by increasing RNA stability, although contrary results were recorded for a chicken lymphoma (Piechaczyk et al, 1985; Rabbitts et al, 1985; Linial et al, 1985). In tumour T24, c-myc RNA levels were not any higher than in tumours where the c-myc transcription unit remained intact (Fig.5.9).

An intriguing possibility is that the T24 insert represents an intermediate in the pathway to myc transduction by FeLV, in accord with models of transduction (Swanstrom et al, 1983). As the insert apparently consists of a 5' FeLV LTR and internal sequences 5' to the gag gene which by analogy with MuLV may include viral RNA packaging signals (Mann et al, 1983), then packageable insert-c-myc fusion transcripts could be generated. To test this hypothesis, I hybridised a Northern blot of tumour T24 RNA with an FeLV U5 probe. Unfortunately, a U5-hybridising band corresponding to the c-myc band could not be identified due to a background smear of U5-hybridising RNA (not shown). However, the possible existence of FeLV-c-myc fusion RNAs could be investigated by cDNA cloning the aberrant c-myc RNA from tumour T24. A similar proviral insertion in a chicken lymphoma was shown to produce a new myc-transducing virus when filtered tumour extract was shown to induce tumours with short latency in young chickens (Robinson & Gagnon, 1986).

5.4.5 Varying ratios of P1 to P2 RNAs in tumour and normal cells

A considerably higher ratio of P1 to P2 RNAs was detected in feline thymic tumours carrying a c-myc rearrangement than in normal fibroblasts or thymocytes, in accord with reports that an elevated P1/P2 ratio may be characteristic of rearranged c-myc genes in human and murine tumours (Table 5.2; Taub et al, 1984a; Hollis et al, 1984; Yang et al, 1985). Tumours T7 and T8 are the first examples where this has been recorded for provir al insertions at the c-myc locus, to extend the previous findings for chromosomal rearrangements.

It might be speculated that enhancers in the LTRs of upstream proviruses preferentially activate transcription of P1 rather than the more downstream P2 promoter, since some enhancers seem to act on proximal rather than distal promoters (review: Lang & Spandidos, 1985).

It is less clear how the 3' rearrangement in tumour T5 might cause a high P1/P2 ratio, although 3' rearrangements in Burkitt's lymphomas have also been reported to be associated with a high P1/P2 ratio (Hollis et al, 1984; Taub et al, 1984b). Perhaps the overall chromatin structure of the locus becomes altered.

My results indicate complexity in control of differential expression of P1 and P2, since several tumours display a high P1/P2 ratio in the absence of detectable c-myc rearrangement. This could result partly from tissue-specific variation (section 5.4.2). It is also possible that as yet undetected point mutations in regulatory elements have the same influence as gross rearrangements on the P1/P2 ratio. If c-myc expression is assumed to be controlled by the products of other genetic loci, then alterations to distant, cis-acting elements or trans-acting factors may disturb the ratio. Similar ideas were invoked to explain the reported elevation of c-myc expression which accompanied rearrangement at the pvt-1 locus, which is >72 kb distant from c-myc, in murine plasmacytomas (Cory et al, 1985; Graham et al, 1985).

	5'		3'
End-labelled Probe/size (bp)	Sma/700	SX2/198	H3B2/645
Strand-separating gel: % polyacryl.	5%	8%	5%
Antisense DNA strand	upper	upper	lower
% GC of DNA/RNA hybrids predicted /size (bp):			
P1	68%/286	69%/138	-
P2	67%/105	-	-
a1	-	-	33%/310
a2	-	-	29%/435
Hybridisation temp. optimum	59°C	59°C	41°C
Analytical gel: % polyacryl.	6%	6%	4%

Table 5.1 Summary of protocols for preparation and use of 5' and 3' end-labelled, single-stranded feline *c-myc* probes for S1 nuclease mapping (section 5.3.1).

Abbreviations:
probes: Sma, SmaI; SX2, Sau3A/XhoII; H3B2, HindIII/BglII.
polyacryl., polyacrylamide
DNA/RNA hybrids predicted: P1, 5' most RNA start site
P2, 3' most RNA start site
a1, 5' most polyadenylation site
a2, 3' most polyadenylation site

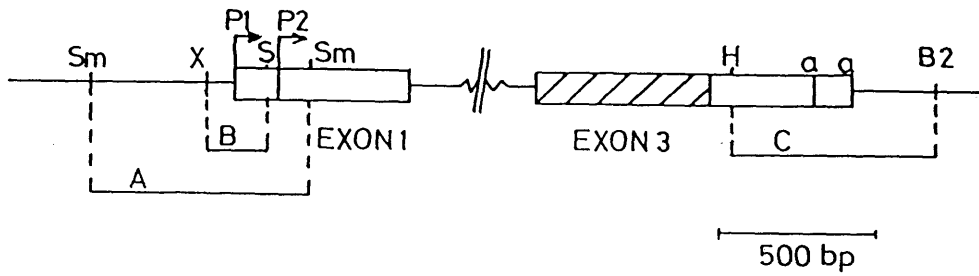


Fig.5.1. Origin of c-myc-derived probes used for S1 nuclease mapping RNA from feline cells.

Probe A encompassed the P1 and P2 promoter-like elements at the 5' end of exon 1, and probe B should be specific for the P1 region. Probe C encompassed both putative polyadenylation signals ("a") at the 3' end of exon 3. Coding sequences are indicated by cross-hatching and non-coding by open boxes.

Restriction enzyme abbreviations: B2, BglII; H, HindIII; S, Sau3A; Sm, SmaI; X, XhoII.

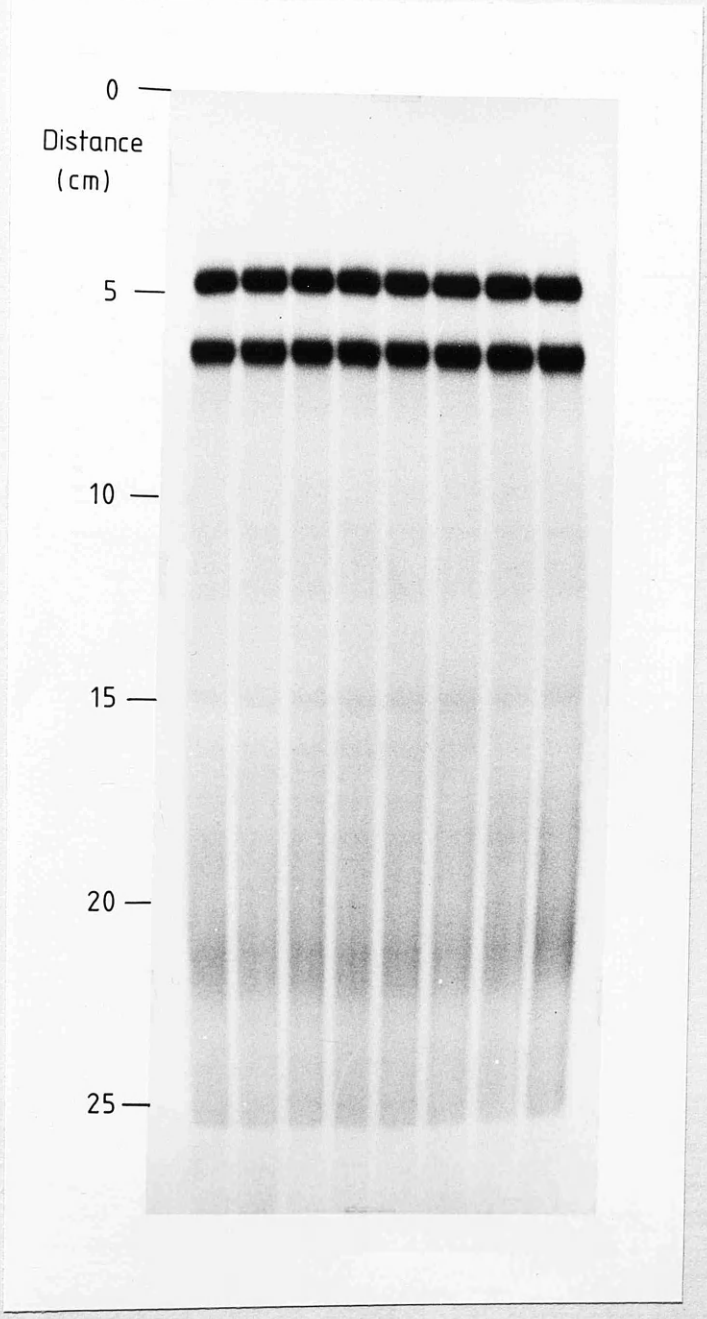


Fig.5.2. Strand-separation of 5' end-labelled probe A.
(Table 5.1; section 5.2.2)

The separated strands of the 5' end-labelled probe A fragment were located on the gel by autoradiography at room temperature for 15 minutes using an intensifying screen.

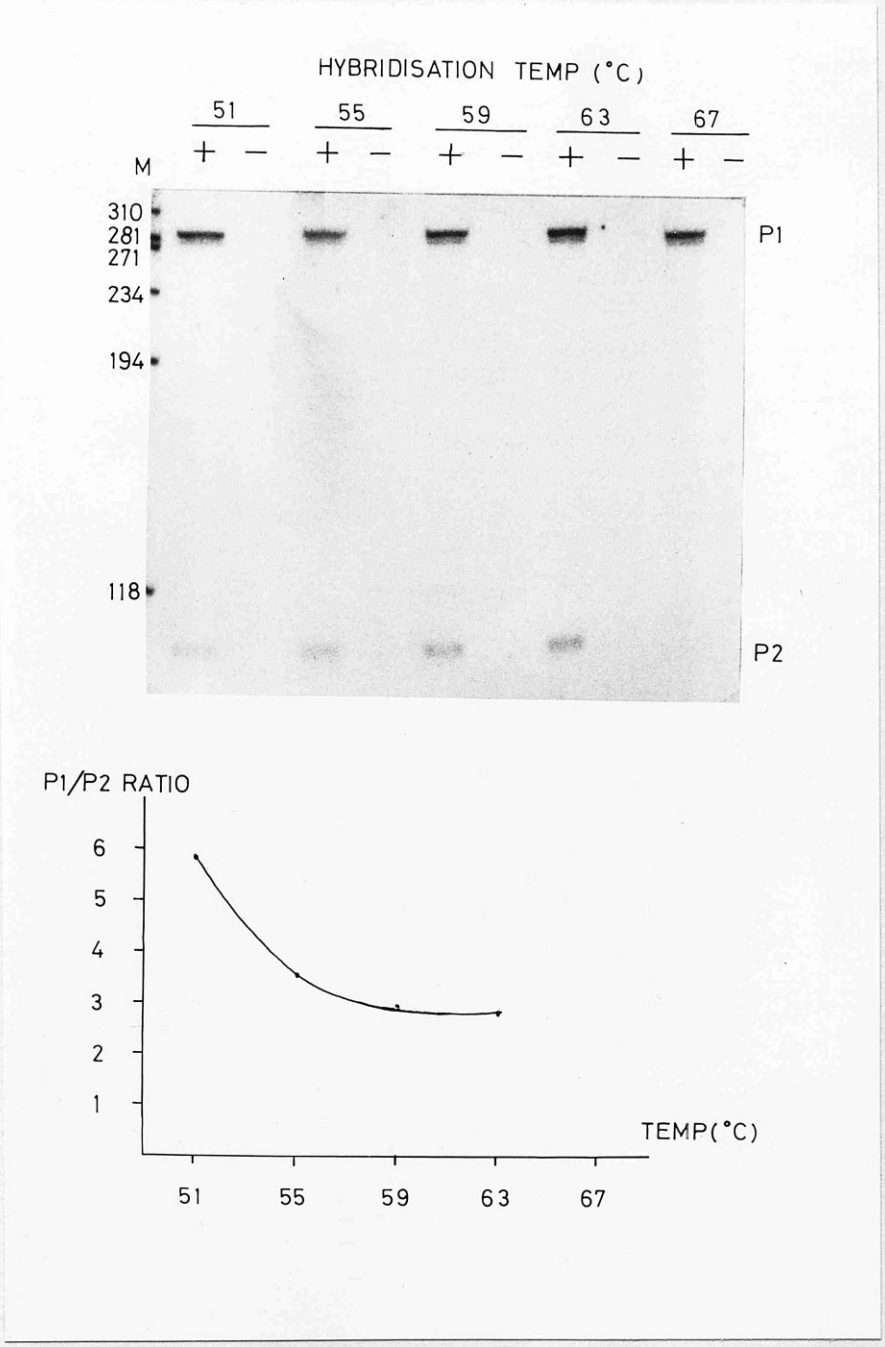


Fig.5.3. Determination of the optimum hybridisation temperature for detection of c-myc P1 and P2 RNA 5' discontinuities using S1 nuclease mapping probe A. (Section 5.3.1; Table 5.1).

The ratio of P1 to P2 hybrids detected for each temperature was quantitated by densitometry, and the results plotted graphically as shown below the autoradiograph. Since in some samples the ratio detected varied greatly, autoradiographs were exposed for the minimum time necessary to permit densitometric analysis of the fainter P2 band. This minimised bias of results due to disproportionate variation of signal intensity with exposure time. +/- = presence/absence of sample RNA.

M, HaeIII-digested ØX174, ^{32}P-labelled markers.

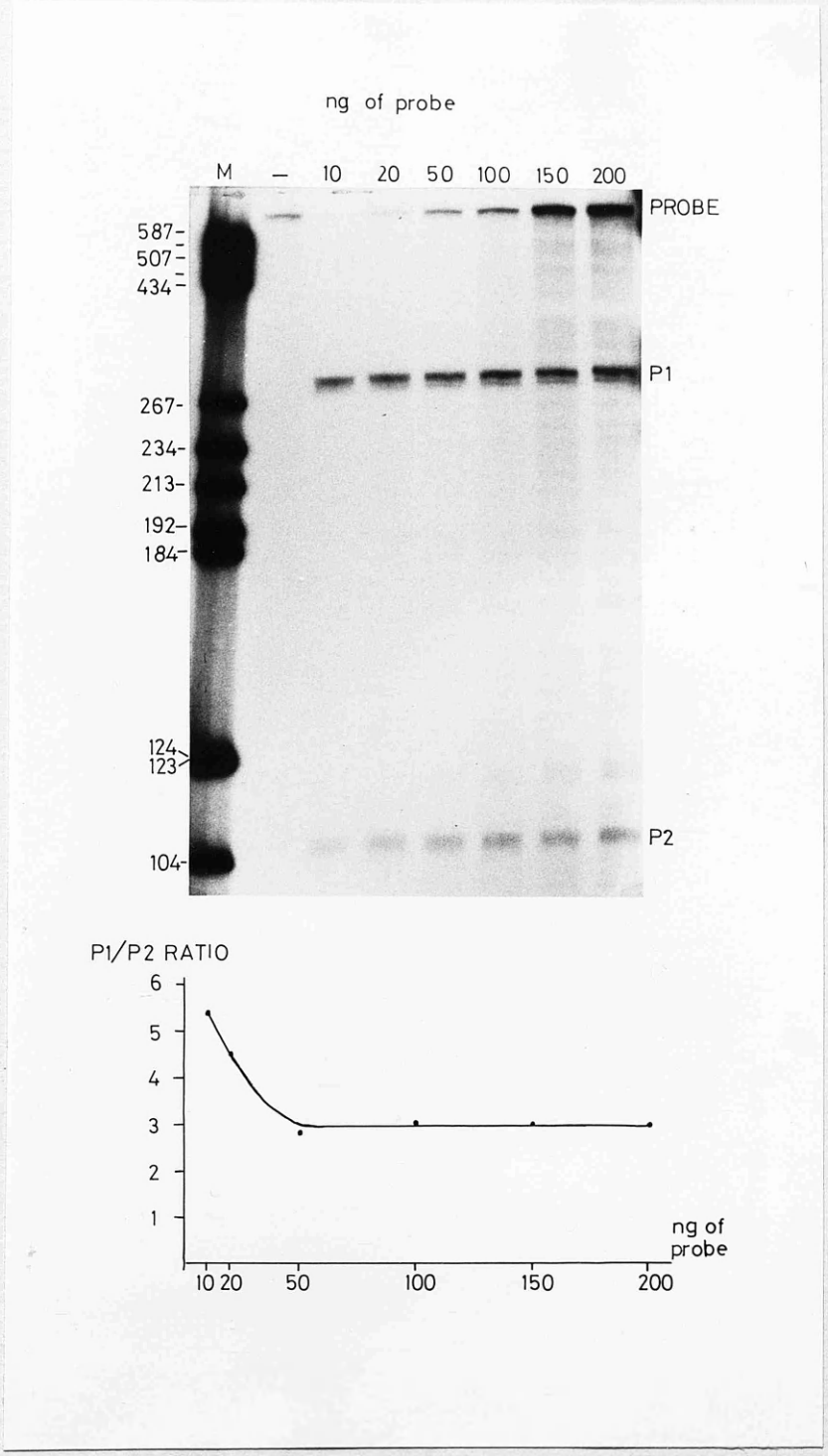


Fig.5.4. Variation in the ratio of c-myc P1 to P2 RNA 5'discontinuities detected by S1 nuclease mapping using different quantities of probe A. (Section 5.3.1; Table 5.1).

The ratio detected increased using < 50ng of probe but was constant with > 50ng of probe, as shown graphically below the autoradiograph.

M, HaeIII-digested pBR322, ^{32}P-labelled markers.

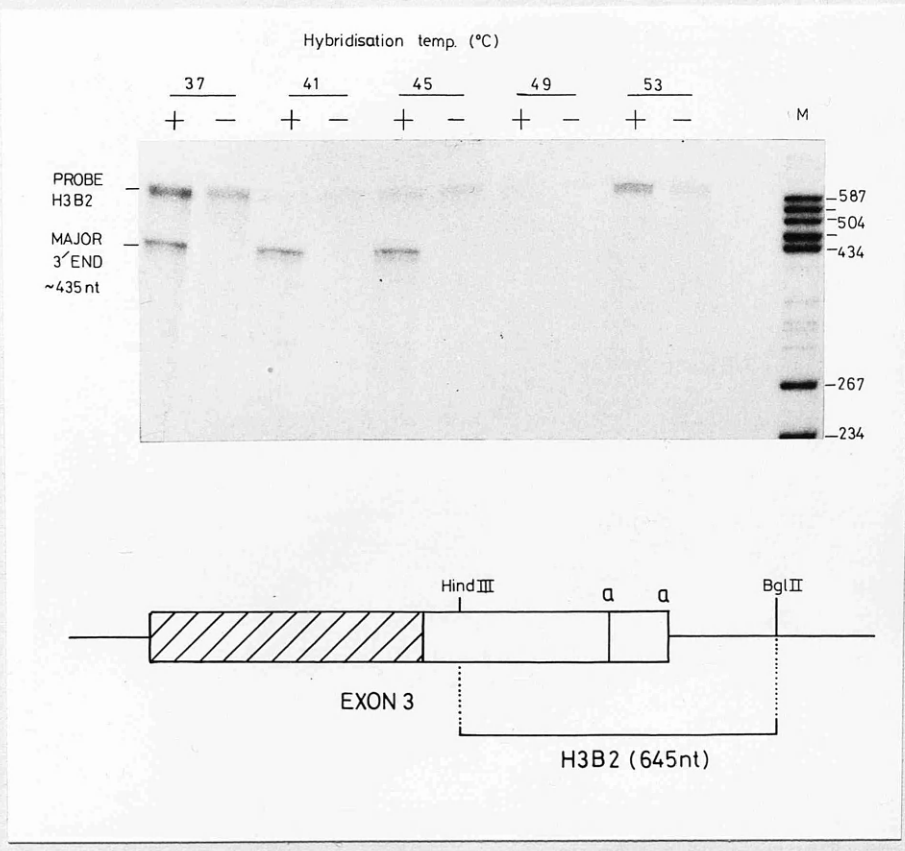
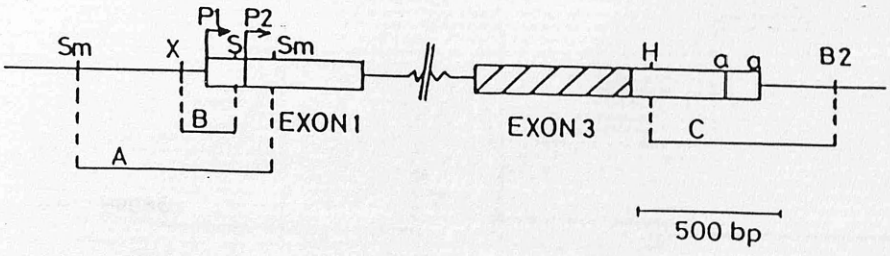


Fig.5.5. Determination of the optimum hybridisation temperature for detection of c-myc RNA 3' discontinuities using S1 nuclease mapping probe C (Section 5.3.1; Table 5.1).

The origin of probe C is shown below the autoradiograph with exon 3 coding regions shown by cross-hatching and non-coding regions by an open box. The %GC base-pairing for RNAs mapping to the two predicted polyadenylation signals ("a") was only ~30%, hence, the optimum hybridisation temperature was determined using a low temperature range.
M, HaeIII-digested pBR322, ^{32}P-labelled markers.



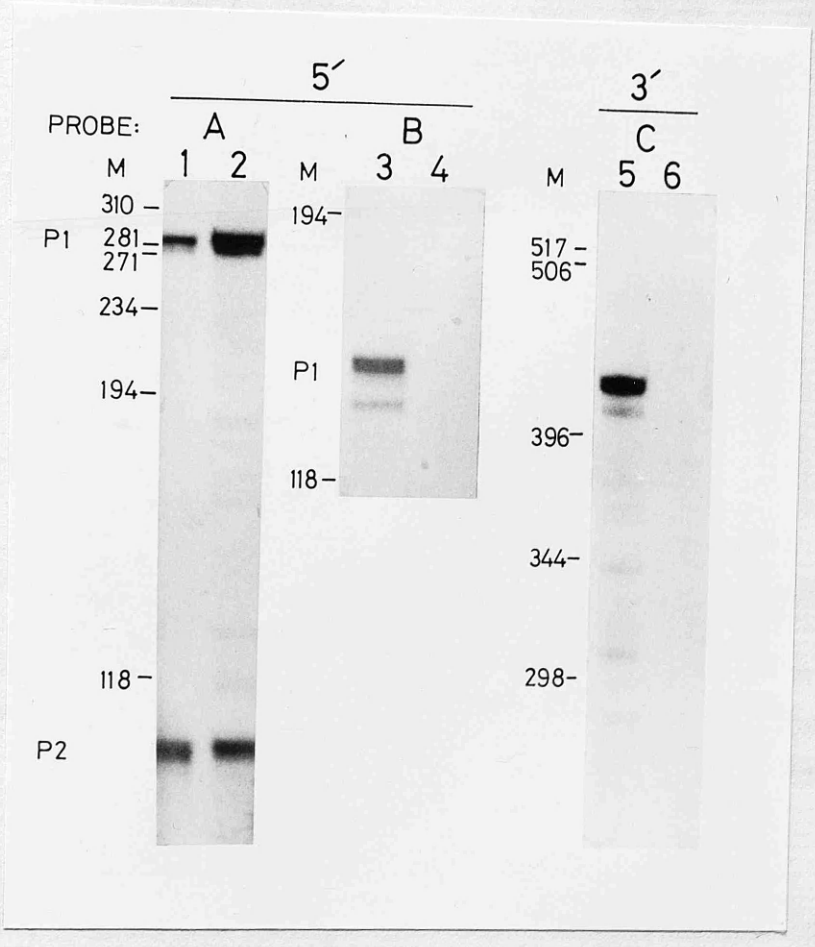
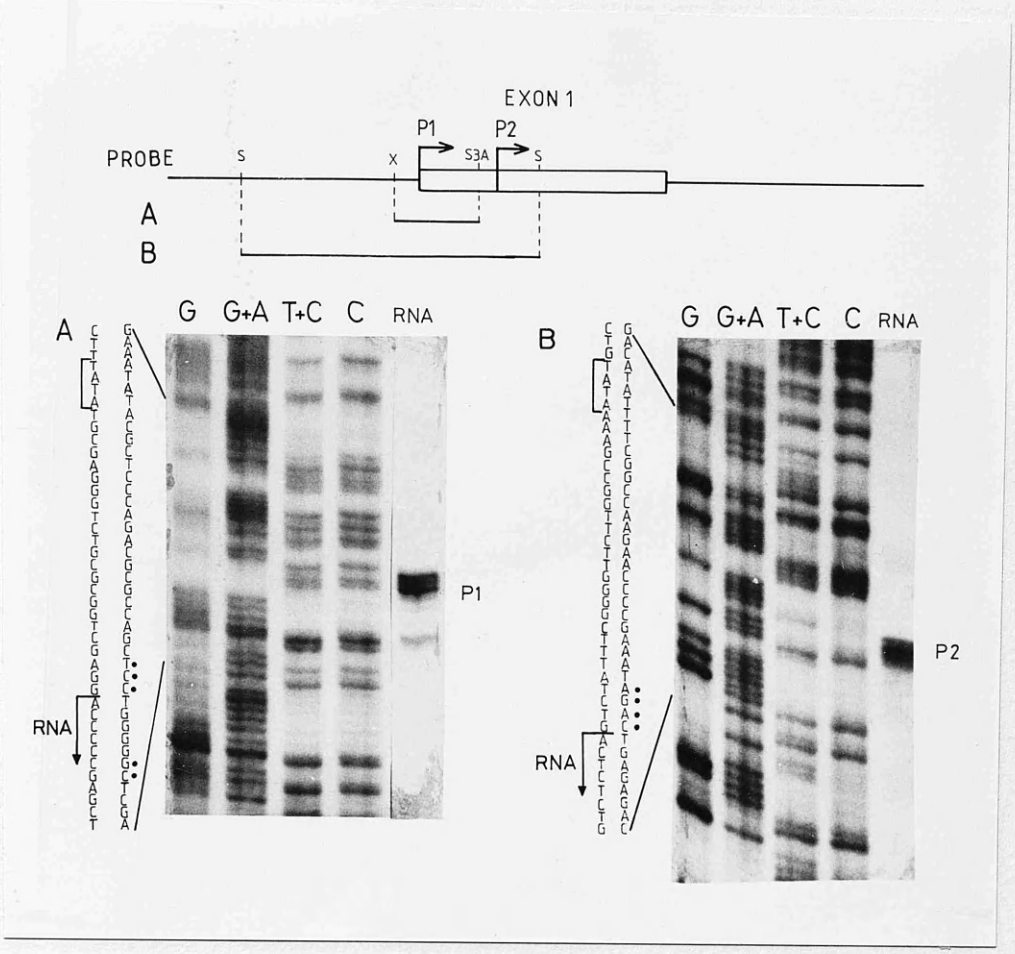


Fig.5.6. Summary of RNA 5' and 3' discontinuities detected with S1 nuclease mapping probes A, B and C (Table 5.1).

A and B show the respective results with 5' end-labelled probes A and B, and C shows results using 3' end-labelled probe C. Lanes: 1 and 5, FEA embryo fibroblast RNA; 2 and 3, tumour case 86800 RNA; 4 and 6, controls with carrier RNA and no sample RNA; M, markers were for A and B, HaeIII-digested ØX174, and for C, HinfI-digested pBR322 labelled with ^{32}P.



+1 RNA

CONSENSUS RNA CAP SITE: G-GTATAA/TAA/T-G--G..9-17bp..Y---YAYYYYYG

FELINE c-myc P1: CTTTATAT GC GAGGG...10bp...CGAGGACCCCCGAGCTGCG

P2: CTGTATAA AA GCCGG...13bp...ATCTGACTCTC

Fig.5.7. P1 and P2 RNA 5' discontinuities detected by S1 nuclease analyses map to consensus promoter sequences.

Top: P1 and P2 hybrids detected with probes A and B in FEA normal embryo fibroblasts (RNA lanes) were electrophoresed alongside chemically-cleaved sequence ladders of probes A and B respectively (lanes G, G+A, T+C, C). To aid interpretation, the origins of the probes are shown at the top, and the sequences of probes A and B are shown to the left of each autoradiograph.

Bottom: The sequences of the P1 and P2 regions are aligned with consensus TATA box and RNA cap site sequences (Breathnach & Chambon, 1981).

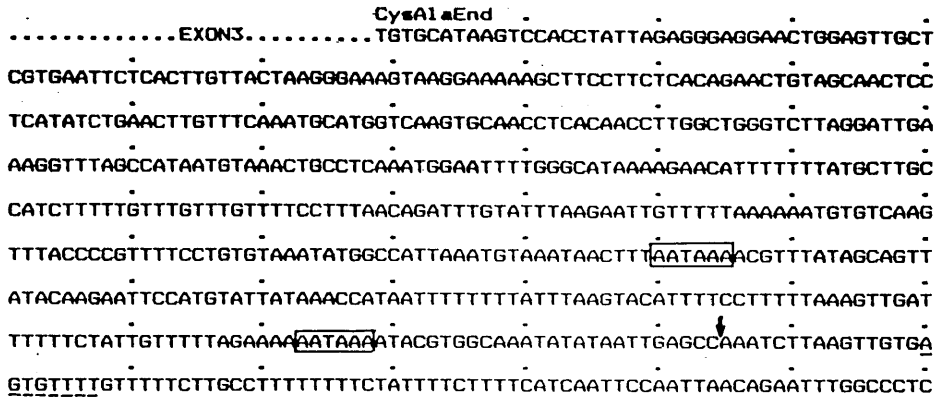


Fig.5.8. Sequence of the 3' end of c-myc exon 3 showing the location of the major RNA 3' discontinuity detected by S1 nuclease mapping.

Consensus polyadenylation signals (AATAAA) are enclosed in boxes and a sequence similar to the consensus YGTGTTYY, where Y = pyrimidine (McLauchlan et al, 1985) is underlined. The location of the 3' RNA discontinuity detected by S1 nuclease mapping is marked by a vertical arrow.

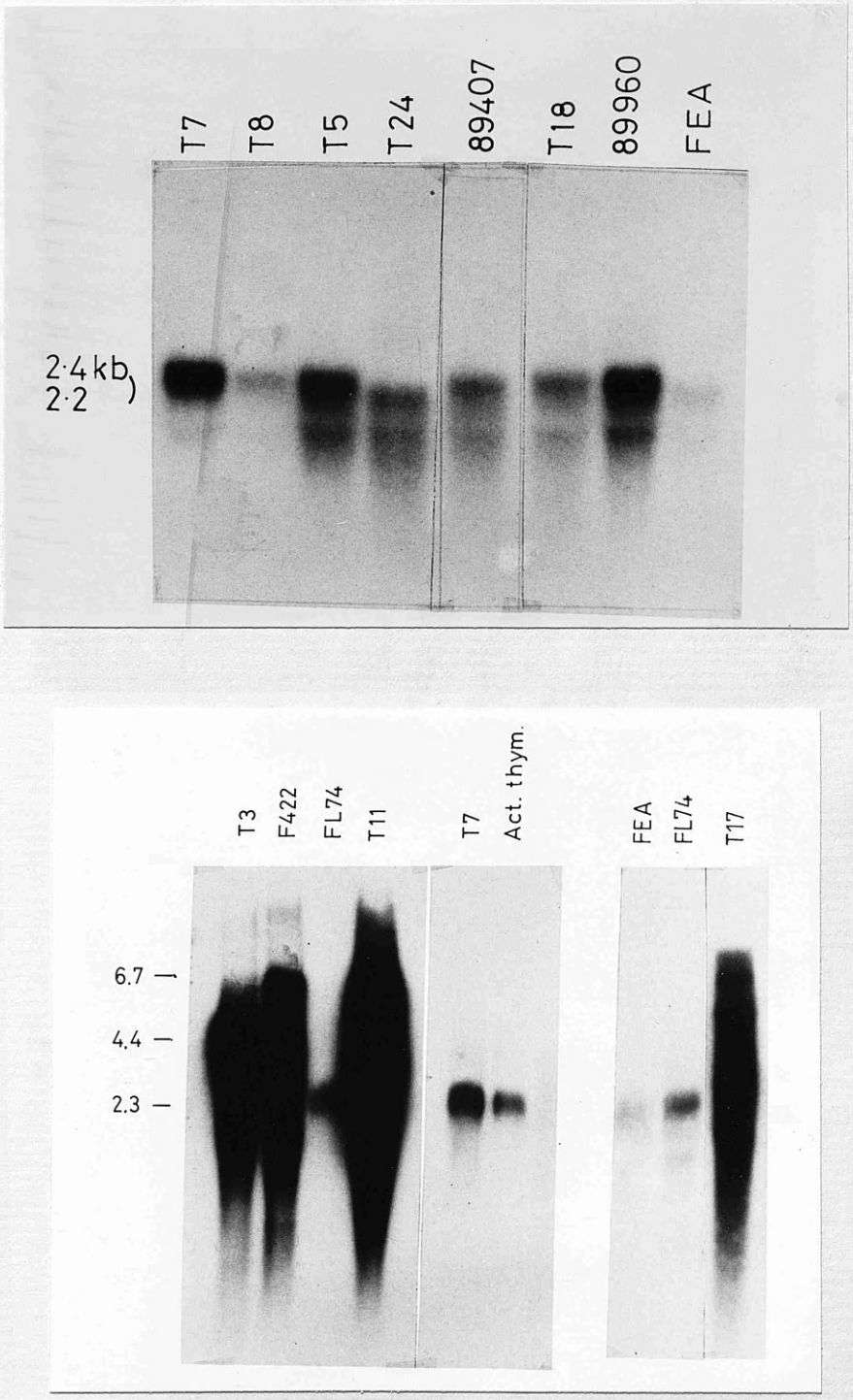


Fig.5.9. Northern blot analyses of RNA from feline tumours and normal cells using an FeLV v-myc probe.

Lanes: tumours carrying a rearranged c-myc gene, T7, T8, T5, T24; tumours containing an FeLV/myc virus, T3, F422, T11, T17; tumours with no apparent myc abnormality, 89407, T18, 89960, FL74; normal cells, FEA embryo fibroblasts, Act. thym., mitogen (ConA) activated normal thymocytes.

Sizes were estimated by comparison with HindIII-digested λDNA, ^{32}P-labelled markers (not shown).

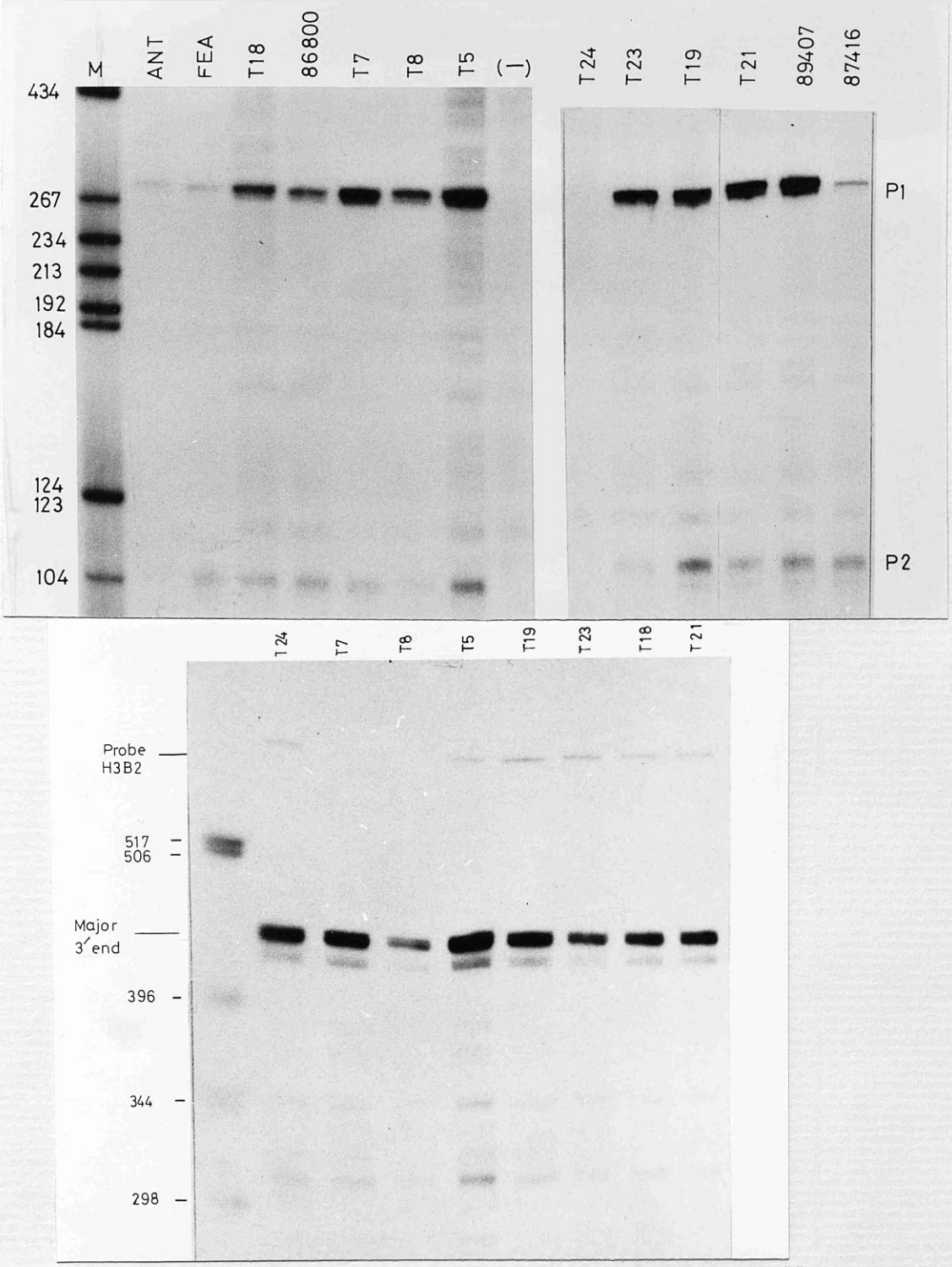


Fig.5.10. S1 nuclease analyses of c-myc RNA in normal cells and in tumours with and without rearrangement of the c-myc locus.

Top: quantitative analysis of the relative amounts of P1 and P2 transcripts, using probe A.

Bottom: Analysis of 3' discontinuities with probe C.

RNA samples were: ANT, mitogen activated normal thymocytes; FEA, normal embryo fibroblasts; T18, 86800, T23, T19, T21, 89407, 87416, tumours with no apparent myc alteration; T7, T8, T5, T24, tumours with a rearranged c-myc locus. A control with carrier RNA but no sample RNA is indicated by (-). M, ^{32}P-labelled markers were top, HaeIII-digested and bottom, HinfI-digested pBR322.

Cells / tissue	FeLV	c-myc rearrangement	P1/P2 ratio[1]
ConA / normal thymocytes	-	-	1.9
FEA embryo fibroblasts	-	-	0.8
T18 thymic LSA[2]	-	-	4.9
86800 " "	+	-	3.1
T7 " "	+	5' insertion	17.9
T8 " "	+	5' insertion	17.0
T5 " "	+	3' alteration	13.6
T24 " "	+	5' insertion	-
T23 " "	-	-	11.8
T19 " "	+	-	7.7
T21 " "	-	-	16.2
89407 " "	+	-	19.6
87416 alimentary LSA	+	-	1.0

Table 5.2. Relative levels of c-myc P1 and P2 transcripts in normal cells and tumours with or without rearrangement of the c-myc locus.

1. P1/P2 ratios were determined by densitometry of autoradiograph bands.
2. LSA = lymphosarcoma.

CHAPTER SIX

ANALYSIS OF EXPRESSION OF v-*myc* AND c-*myc* GENES IN TUMOURS CONTAINING A *myc*-TRANSDUCING FeLV.

6.1 INTRODUCTION

FeLV/*myc* recombinant viruses have been identified in a subset of field cases of feline thymic lymphosarcoma (Neil et al, 1984; Levy et al, 1984; Mullins et al, 1984). To date, seven independent isolates have been identified (Table 6.1). The pathogenic properties of two isolates (FeLV-GT3 and -FTT) have been investigated in Glasgow (D. Onions et al, in press). Both viruses induced thymic tumours 12-14 weeks after intra-peritoneal inoculation in neonatal kittens, indicating that FeLV/*myc* viruses can be causal agents of this disease. The disease onset was rapid compared to the usual latency of more than one year characteristic of inoculation of non-oncogene carrying FeLVs isolated from cats without tumours (D. Onions, pers. comm.). Even with the highly pathogenic FeLV Rickard strain which does not appear to carry an oncogene, lymphosarcomas do not develop for about six months (Rickard et al, 1969). The FTT isolate has also been shown to be active in *in vitro* transformation of primary rat fibroblasts when co-transfected with the EJ-*ras* gene (D. Doggett et al, submitted).

Considerable study has been made of the structure and expression of the v-*myc* genes of well-characterised avian acute leukaemia viruses (Bister, 1984). However, there has been little investigation of the molecular biology of the v-*myc* genes of the more recently discovered FeLV/*myc* viruses, which represent the only examples of transduction of the mammalian *myc* gene. To begin to address this issue, the sequence of the three FeLV v-*myc* genes has been determined (Chapter 3). This chapter investigates another aspect: the structure and expression of transcripts of feline v-*myc* genes.

When this study began, observations on expression of c-*myc* alleles which had been rearranged by chromosomal translocation and of normal c-*myc* alleles in Burkitt's lymphomas led to the proposal of a model of autoregulatory control of expression of the c-*myc* gene (Leder et al, 1983; Rabbitts et al, 1984). During my study I recorded some results which similarly bear on control of c-*myc* expression. These results extended the earlier reports as my work was based on analysis of

transduced v-myc genes and normal c-myc genes in feline T-cell tumours.

6.2 RESULTS

Evidence that some feline thymic tumours contain a myc-transducing FeLV was first based on Southern blot data. Further proof in three tumours was provided in the form of molecular clones of the FeLV/myc proviruses CT4, LC and FTT (Table 6.1). For tumour T3, purified virion RNA from cell culture supernatant has been shown to hybridise to both myc and exogenous FeLV U3 probes (J. Neil, pers. comm.).

6.2.1 Expression of v-myc genes

Fig.6.1 shows Northern blot analyses using a v-myc probe, of RNA from tumours containing FeLV/myc viruses GT3, GT11, FTT and GT17 alongside normal cells and control tumours which do not contain an FeLV/myc virus. Levels of myc-hybridising RNA were dramatically elevated in cases with an FeLV/myc virus. It has been established that these highly expressed bands represent v-myc transcripts in cases containing viruses GT3 and FTT, by hybridisation analysis of purified virion RNA with a myc probe (J. Neil, pers. comm.). In tumours containing viruses GT11 and GT17, in the absence of further evidence, the unusual size and high level expression of the myc-hybridising RNA strongly suggested that it derived from the GT11 and GT17 proviruses.

6.2.2 Structures of FeLV v-myc transcripts

Northern blot analysis indicated the sizes of the largest, presumably genomic, v-myc RNAs produced by FeLV-GT3, GT11, GT17 and FTT to be approximately: GT3, 5.0 kb; GT11, 7.5 kb; GT17, 6.0 kb and FTT, 7.0 kb (Fig.6.1). In the case of FTT this agreed with the size of the proviral molecular clone (D. Doggett et al, submitted). For tumour F422, shorter autoradiographic exposure identified a 3 kb band corresponding to a sub-genomic v-myc RNA predicted from sequence data, since the FTT myc insert is downstream from the env splice acceptor signal (D. Doggett et al, submitted). Similar analysis identified a single 5 kb band in RNA from tumour T3 suggesting that the GT3 v-myc gene is not expressed from a sub-genomic RNA. This agrees with immunoprecipitation studies using FeLV gag antisera, which identify a novel gag protein in tumour T3 which may represent a gag-myc fusion product (J. Neil, pers. comm.).

6.2.3 Truncation of exon 1 in FeLV v-myc genes

All sequenced FeLV v-myc genes contain truncated exon 1 sequences (Chapter 3). S1 nuclease mapping with probe A of RNA from tumours containing the GT3 virus indicated that its v-myc gene included at least 90 nucleotides upstream from the SmaI site shown in Fig.6.2. However, it did not include sequences upstream of the Sau3A site 5' to the P2 region as no signal was detected with probe B (Fig.6.3). The band was defined as viral rather than cellular since the 90 nucleotide band was detected in all GT3-containing tumours but not in any others. The GT11 and GT17 viral transcripts did not contain exon 1 sequences which could be detected with probe A (Fig.6.2).

The GT3 virus contained an unusually large portion of exon 1 compared to other studied FeLV v-myc genes. It would be of interest to know if this provided a novel polypeptide domain fused to the normal c-myc N-terminus. Confirmation of this would require cloning of the GT3 provirus.

6.2.4 3' termini of transduced myc sequences

Fig.6.4 summarises the location of the 3' termini of the transduced myc sequences of four viruses. The termini of the FeLV-FTT and -LC isolates were respectively 12 and 99 nucleotides 3' to the exon 3 stop codon. The myc 3' ends of the GT3 and GT11 viruses appeared to be ~340 nucleotides 3' to the exon 3 stop codon based on S1 nuclease mapping (Fig.6.3). Until the GT3 and GT11 viruses may be cloned, it cannot be excluded that the transduced myc sequences extend further 3' than this, but have acquired a mutation at this site causing an S1 nuclease-sensitive mismatch to the probe. For the GT3 virus the band was defined as viral rather than cellular since it was present in all studied tumours containing FeLV-GT3. The GT11 v-myc gene seemed to terminate at the same site. In all cases (FeLV-LC, -FTT, -GT3, -GT11), the transduced myc sequence terminates in the region between the c-myc exon 3 stop codon and the 5' most polyadenylation signal.

6.2.5 Lack of detectable RNA from normal c-myc alleles in tumours containing an FeLV/myc virus

High level v-myc expression obscured detection of RNA from the c-myc gene by Northern blot analysis (Fig.6.1). However, it was possible to investigate the presence of c-myc RNA by S1 nuclease analysis as no known FeLV v-myc gene contains entire exon 1 or exon 3 untranslated

sequences, facilitating distinction between c-myc and v-myc transcripts (see above). Tumours and tumour cell lines involving four different FeLV/myc viruses were analysed (Table 6.1). The tumours in which the viruses were identified have been shown to express high levels of v-myc transcripts, although this has not yet been demonstrated for all additional tumours induced by the GT3 and FTT viruses.

Consistently, in tumours containing an FeLV/myc virus, no c-myc RNA could be detected using probes A, B or C (Fig.6.2). The RNA samples had been shown to be intact by Northern blot analyses for expression of T-cell antigen receptor genes (Chapter 7). Using probe A, tumours containing the GT3 virus displayed an internal positive control band , which as discussed above represents a GT3 v-myc exon 1 sequence. As expected from the sequence of the FTT v-myc gene (D. Doggett et al, submitted), probe A did not detect any S1 nuclease resistant bands in tumours containing this virus, as the probe did not encompass exon 1 sequences present in FeLV-FTT.

Analysis with probe B showed that lack of detectable c-myc RNA in tumours containing FeLV-GT3 was not due to saturation of probe A with a vast excess of v-myc transcripts (Fig.6.3). Probe B should specifically detect c-myc P1 RNA without competition from v-myc RNA, but again no v-myc or c-myc P1 RNA was detected.

Analysis with probe C also showed that c-myc RNA could not be detected (Fig.6.3). Again tumours containing the GT3 virus displayed an internal positive control band representing transduced c-myc 3' untranslated sequence as discussed above. Tumour T11 displayed a similar band. In tumours containing FeLV-FTT and -GT17, no bands were detected. Since probe C correponded to only 12 nucleotides of the 3' end of the v-myc gene of FeLV-FTT, we would not expect to detect such a small S1 nuclease-protected hybrid under the conditions used in this analysis. The same explanation could apply for the GT17 v-myc gene which has not been sequenced.

6.3 DISCUSSION

6.3.1 Expression of v-myc genes in thymic lymphosarcomas

A consistent feature of tumours in which four different FeLV/myc viruses have been identified is that high levels of v-myc transcripts are present. This suggests that v-myc transforming activity may be

exerted through over-expression of the product, but does not discount the possible role of structural alterations in the myc product (Chapter 3). High level v-myc transcription would be driven by powerful FeLV LTR controls, since no studied FeLV v-myc gene retains the normal c-myc promoters. Tumours containing the FTT and GT3 viruses may give rise to transcripts from several different copies of the v-myc gene, since these tumours carry three to six integrated FeLV/myc proviruses (Neil et al, 1984; Mullins et al, 1984; D. Onions et al, submitted).

No known FeLV v-myc gene contains an intact c-myc exon 1 (section 6.2.3). Therefore these may lack sequences which normally confer the very short half-life of the c-myc RNA (Dani et al, 1984), perhaps increasing v-myc RNA levels through greater stability. Deletion of exon 1 in rearranged human and mouse c-myc genes has been reported to stabilise c-myc RNA (Piechaczyk et al, 1985; Rabbitts et al, 1985).

Transduced myc sequences appear to terminate upstream of the c-myc polyadenylation signals in all studied FeLV v-myc genes (Fig.6.4). This may reflect the requirement to prevent premature termination of transcription of the recombinant proviruses.

6.3.2 Loss of detectable transcripts from normal c-myc alleles

Eight tumours and tumour cell lines involving four different FeLV/myc viruses (Table 6.1) were found to lack detectable c-myc RNA using S1 nuclease mapping probes which should detect normal c-myc RNA 5' and 3' ends. A possibly analogous result was recorded in tumour T24 which carries an insertion in c-myc intron 1, where RNA derived from the rearranged but not the normal allele could be detected (Chapters 4 and 5). Thus, RNA could not be detected which derives from the single normal c-myc allele in tumour T24 or from either of the two ostensibly normal c-myc alleles in tumours containing a myc-transducing FeLV.

The level at which c-myc expression is being down-regulated cannot be determined by these studies since S1 nuclease analyses performed on steady-state total cellular RNA do not rule out post-transcriptional control. However, lack of detectable c-myc RNA using a sensitive S1 nuclease analysis strongly suggests that transcriptional control is of prime importance. Further support for this view would require transcriptional run-off assays on isolated nuclei, or analysis of DNAseI hypersensitive sites at the c-myc loci.

It has similarly been recorded that in mouse lymphoid cells and fibroblasts infected with, and in tumours induced by, recombinant MuLVs which express an avian v-myc construct, c-myc RNA was not detected (Rapp et al, 1985; Morse et al, 1986). Likewise it has been reported that in human and mouse B-cell lymphomas with a translocated c-myc gene, the rearranged allele is expressed but the normal allele is not or is expressed at very reduced levels (ar-Rushdi et al, 1983; Bernard et al, 1983; Rabbitts et al, 1984; Taub et al, 1984a; Fahrlander et al, 1985b). Also, in tumours in transgenic mice which carry and express a c-myc construct, and in tumours derived from fibroblasts which carry a c-myc construct, normal endogenous c-myc RNA could not be detected (Adams et al, 1985; Keath et al, 1984).

In general, expression of the c-myc gene occurs in growing cells, being elevated upon growth stimulation and reduced with growth inhibition (Kelly et al, 1983; Reed et al, 1985; Reitsma et al, 1983; Lachman & Skoulchi, 1984). Hence, it is intriguing that actively growing feline T lymphocytic tumour cells which carry an FeLV/myc virus contain no detectable RNA from the endogenous c-myc gene. The consistent association between the presence of any one of four different FeLV v-myc genes and the lack of detectable c-myc RNA suggests that down-regulation is related to expression of the v-myc genes perhaps in accord with the proposal that expression of the c-myc gene is under negative feedback control mediated directly or indirectly by the myc product (Leder et al, 1983; Rabbitts et al, 1984). It would be possible to test this idea directly in the feline system by manipulation of the myc coding sequence in cloned FeLV/myc viruses to investigate the role of the myc product in this phenomenon. As virtually nothing is known of the normal function of the myc product, it can only be speculated that loss of normal c-myc expression directly contributes to transformation or instead is only coincidental to the transformation process mediated by v-myc genes.

FeLV/*myc* virus involved	Tumour case	Primary tumour (P) or tumour cell line (L)	Tumour of origin of virus or tumour induced by virus	Proviral molecular clone	Ref.
FeLV-CT4	84793	P	origin	+	1
FeLV-C1	84929	P	origin	-	1
FeLC-LC	1110	P	origin	+	2
FeLV-GT11	T11	P	origin	-	1
FeLV-GT17	T17	P	origin	+	5
FeLV-GT3	T3	L	origin	-	1
"	GT3-C4	P	induced	-	3
"	GT3-C8	L	induced	-	3
FeLV-FTT	F422	L	origin	+	4
"	F422-1	P	induced	*	3
"	F422-3	P	induced	*	3

Table 6.1. Characteristics of thymic tumours which contain a *myc*-transducing FeLV.

* Although a molecular clone of FeLV-FTT exists, virus stocks from the original tumour cell line F422 were used to induce tumours F422-1 and F422-3 (ref. 4).
Refs. 1, Neil et al, 1984; 2, Levy et al, 1984; 3, D. Onions et al, in press; 4, Mullins et al, 1984; 5, bacteriophage λ clone, R. Fulton, pers. comm..

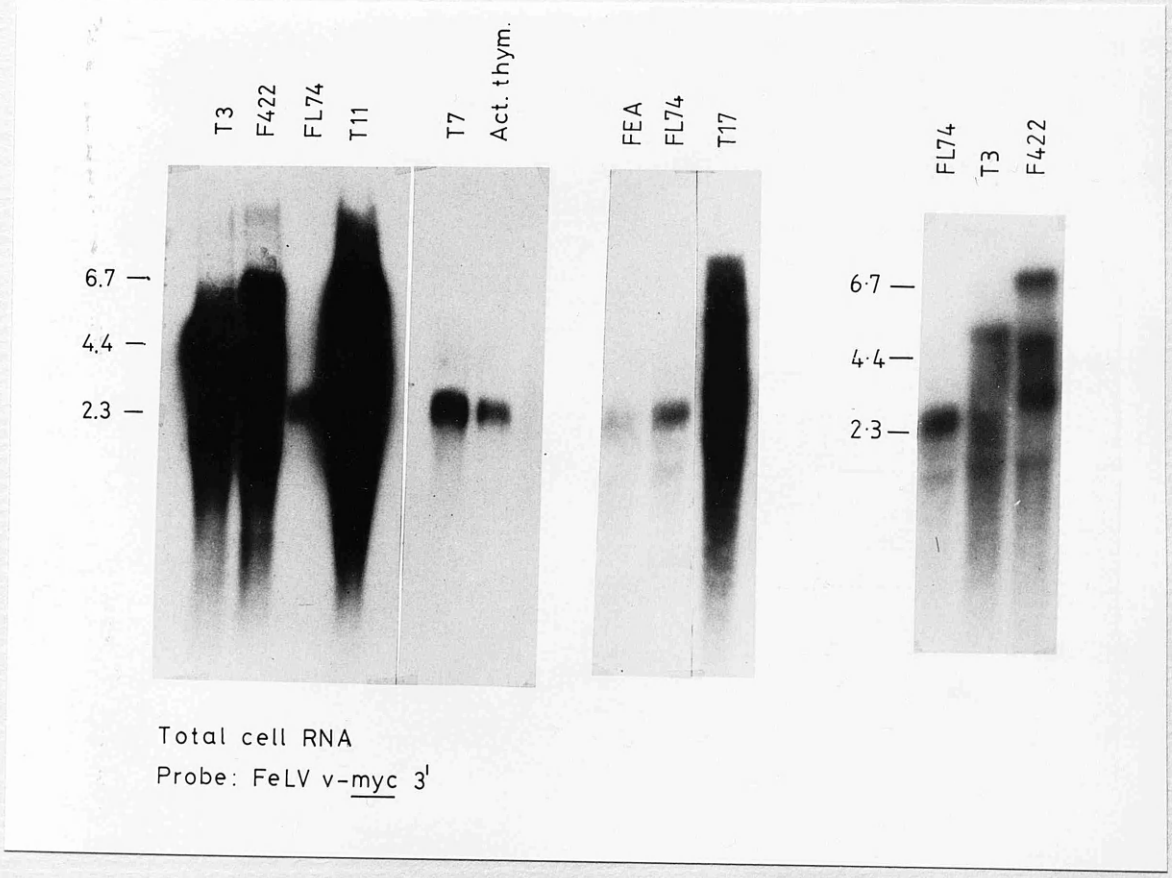


Fig.6.1. Northern blot analyses using a v-myc probe of RNA from tumours.

RNAs were from tumours containing an FeLV/myc virus (T3, F422, T11, T17), a c-myc rearrangement (T7) or no myc alteration (FL74), and normal embryo fibroblasts (FEA) and ConA activated normal thymocytes (Act. thym.).

All lanes contained 20µg of total cellular RNA except for lanes T3 and F422 in the right panel which contained 4µg samples. All autradiographs were exposed to film overnight, except for the right panel which was exposed for 6h. The probe was derived from the pCT4 clone (Fig.4.1).

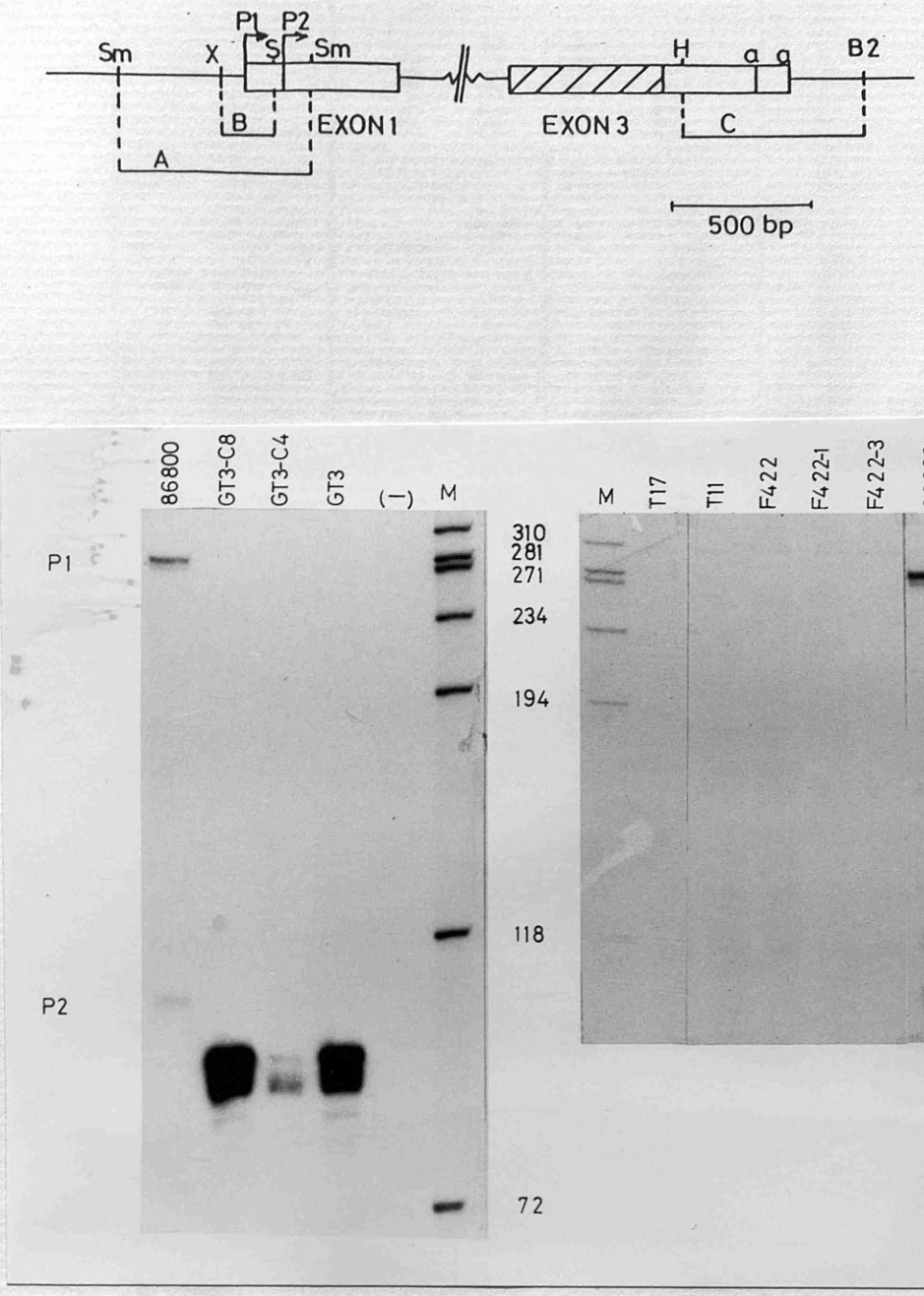
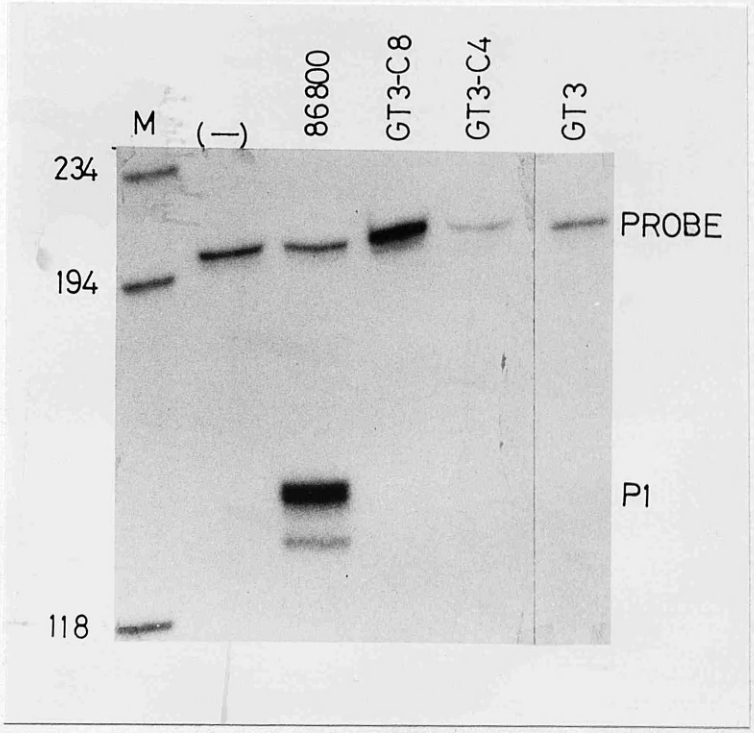


Fig.6.2. S1 nuclease analysis of RNA from tumours containing an FeLV/myc virus.

Top: The origins of probes A, B and C used in the studies shown in Figs.6.2 and 6.3 were the same as described in Fig.5.1. Restriction enzyme abbreviations: B2, BglII; H, HindIII; S, Sau3A; Sm, SmaI; X, XhoII.

Bottom: Results of analysis using probe A of tumours containing FeLV/myc viruses and of tumour 86800 with no detectable myc alteration. (-) represents a control with only carrier rRNA and no sample RNA.
M, HaeIII-digested ØX174, ^{32}P-labelled markers.



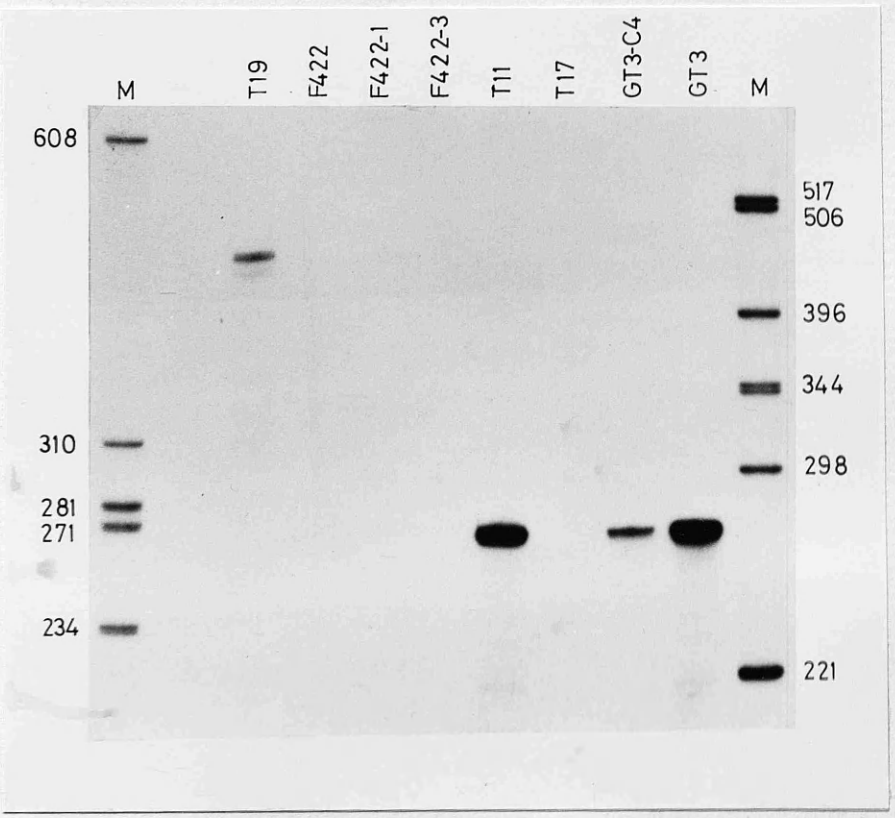


Fig.6.3. S1 nuclease analysis using probes B (top) and C (bottom) of RNA from tumours containing an FeLV/myc virus.

All samples and marker lanes are as described in Fig.6.2 except tumour T19 which has no detectable myc alteration. The right hand marker lane of panel C contained HinfI-digested pBR322, labelled with ^{32}P.

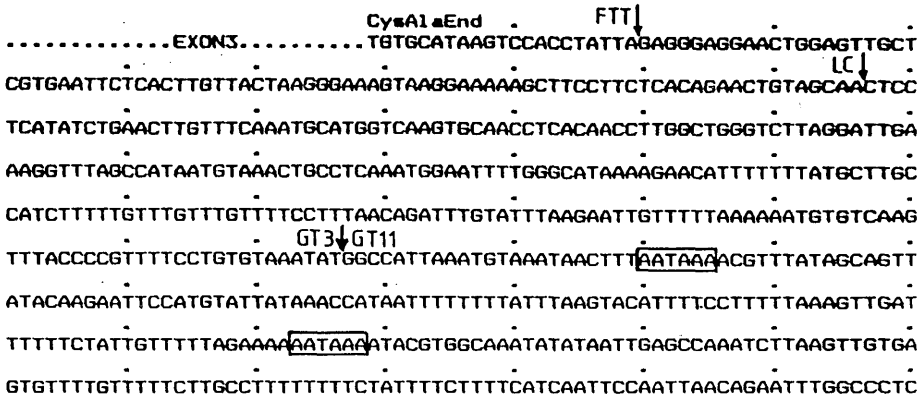


Fig.6.4. Location of the 3' termini of transduced myc sequences in FeLV/myc viruses.

Consensus polyadenylation signals are shown in boxes, and the 3' v-myc termini are indicated by vertical arrows above the sequence. The FeLV-FTT and LC 3' termini were based on sequence data (Braun et al, 1985; D. Doggett et al, submitted) and those of FeLV-GT3 and GT17 on S1 nuclease analyses (Fig.6.3).

CHAPTER SEVEN

EXPRESSION OF T CELL ANTIGEN RECEPTOR GENES

7.1 INTRODUCTION

Most of the tumours available for study in the Glasgow series were field case thymic lymphosarcomas, although some other haemopoietic tumour types were represented. Although on pathological examination the tumours were thymic in origin, it has only recently become possible, following the cloning of genes for various T-cell marker molecules, to characterise their phenotype at a finer level. This study approached several issues concerning lymphosarcoma development. For example, expression of T-cell markers would provide strong evidence that the tumours were of T-cell origin. It would also be interesting to ask if the phenotype of the tumours in which the GT3 and FTT viruses were identified was reproduced in tumours induced by these viruses. This would help define the transforming spectrum of FeLV/myc viruses.

The differentiation stages of human and murine T-cells have been characterised with series of monoclonal antibodies (Reinherz et al, 1980). More recently, cDNA clones have been isolated for components of the T-cell antigen receptor (TCR) and various T-cell differentiation markers which define T-cell subsets (Collins & Owen, 1985; Parnes, 1986). Use of these clones as hybridisation probes has facilitated analysis of T-cell developmental pathways. Thus, it is apparent that prior to expression, the α- and β-chain genes of the TCR rearrange in a similar fashion to immunoglobulin genes in B-cell development (Hood et al, 1985). Expression of TCR genes appears to occur relatively late in T-cell development, with that of the β-chain preceding that of the α-chain (Collins et al, 1985; Furley et al, 1986).

I undertook initial characterisation of the series of feline tumours by Northern blot analysis using probes from cDNA clones of the human TCR α- and β-chain genes (Collins et al, 1985).

7.2 RESULTS

7.2.1 Detection of TCR α- and β-chain transcripts in thymic tumours

Northern blot analysis with the TCR α-chain probe detected a band of 1.6 kb and the β-chain probe detected a band of 1.4-1.6 kb in RNA from thymic tumours and normal thymocytes (Fig.7.1). These sizes are

similar to α- and β-chain RNAs detected in human and mouse T-cells (Yoshikai et al, 1984; Collins & Owen, 1985). The diffuse β-chain band could in some cases be resolved into two bands (Fig.7.2, lane T3). By analogy with human T-cells, the shorter β-chain RNA probably results from incomplete rearrangement of one of the TCR β-chain alleles. In some cases a fainter 4.5 kb band was detected which may represent a precursor of the mature β-chain RNA. The specificity of the probes was demonstrated in that thymic tumours and normal thymocytes displayed strong α- and β-chain bands, whereas only a faint background smear was detected in embryonic fibroblasts and in normal kidney and spleen tissues. Tumour FL74 did not contain detectable β-chain RNA but appeared to express low levels of α-chain RNA.

7.2.2 Expression of TCR α- and β-chain transcripts in tumours containing FeLV/myc viruses.

Fig.7.2 shows that tumours in which FeLV/myc viruses were discovered, and additional tumours induced by FeLV-GT3 and FTT, expressed both α- and β-chain RNAs. Tumour T17 in which FeLV-GT17 was identified, expressed a normal α-chain transcript but displayed gross elevation of an abnormally large β-chain transcript.

7.3 DISCUSSION

7.3.1 Expression of TCR α- and β-chain RNAs in thymic tumours

Several thymic tumours studied produce TCR α- and β-chain RNAs. Some tumours have also been shown to contain rearranged TCR β-chain genes (J. Neil and R. McFarlane, pers. comm.). Rearrangement normally precedes expression in human and murine T-cells (Collins & Owen, 1985). Tumours expressing α- and β-chain RNAs would be of relatively mature phenotype by analogy with human T-cells where TCR expression occurs late in differentiation (Collins et al, 1985; Royer et al, 1985).

However, not all cases showed rearrangement of the β-chain gene indicating some phenotypic heterogeneity. Interestingly, the leukaemic FL74 line seemed to express only α-chain RNA suggesting that it may represent a less mature T-cell tumour. A study of several AKR mouse thymic lymphoma lines showed that many expressed normal α- but not β-chain RNA, and may represent a distinct T-cell subset (Owen et al, 1986).

7.3.2 The phenotype of tumours induced by FeLV/myc viruses

Avian myc-transducing viruses induce a wide spectrum of tumours including various leukaemias and carcinomas in chickens (Hayman, 1983). Also, recombinant MuLVs carrying an avian myc construct induce a range of haemopoietic and epithelial tumours in NFS/N mice (Morse et al, 1986), although another MuLV/avian v-myc construct has been found to induce lymphoid leukaemias and T-cell lymphomas in NIH Swiss mice (Brightman et al, 1986). In contrast, tumours induced by FeLV/myc viruses have been found consistently to be thymic lymphosarcomas, expressing TCR α- and β-chain RNA. Similarly, feline thymic tumours with a rearranged c-myc gene which were studied also express TCR α- and β-chain genes (cases T7, T8, T5).

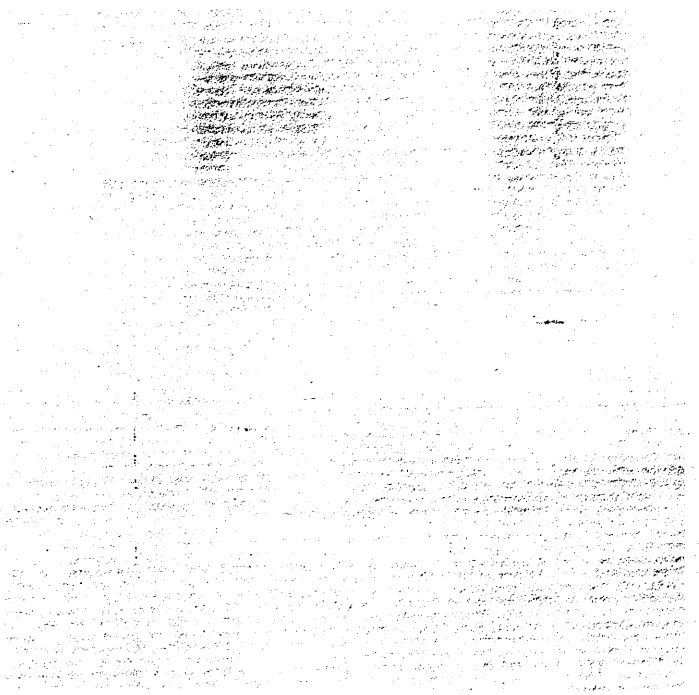
The mono- or oligoclonal nature of the tumours induced by FeLV/myc viruses suggests that not all cells which are infected become transformed, but only those rare cells which acquire a secondary transforming event. The nature of such additional events is not clear at present but the restricted phenotype of the thymic tumours raises intriguing possibilities. This restriction may reflect a requirement for the molecular events associated with rearrangement and expression of the TCR genes, or for the expression of a functional antigen receptor. Thus, aberrant recombination events could activate cellular oncogenes, as may be the case with abnormally joined c-myc and TCR α-chain genes in some human T-cell leukaemias (Erikson et al, 1986; Shima et al, 1986).

It is also possible that T-cells which express functional antigen receptors and hence are responsive to antigenic growth stimuli, are most susceptible to transformation by FeLV/myc viruses or rearranged c-myc genes. This proposal could be in accord with the model of McGrath et al (1980) for murine T-cell lymphomagenesis. This suggests that MuLV-infected T-cells are subject to repetitive mitogenic stimulation of their antigen receptors by viral env products. Perhaps feline T-cells which carry an altered myc gene and which express a functional TCR specific for viral antigens may be selected in tumour development.

7.3.3 Identification of a TCR β-chain gene transduced by FeLV

This study uncovered one apparently exceptional case of involvement of TCR genes in feline thymic lymphosarcoma. In tumour T17 the highly expressed, abnormally large β-chain RNA was reminiscent of the pattern

of expression of a transduced myc gene from an FeLV/myc provirus (Chapter 6). Southern blot analysis with the β-chain probe supported the possibility that this tumour contained an FeLV provirus which had transduced a TCR β-chain gene and this has since been confirmed by isolating a clone of the provirus (R. Fulton, D. Forrest, R. McFarlane, D. Onions and J. Neil, in press). Therefore this tumour contained two independently transduced cellular genes: v-myc and v-tcr (T-cell receptor β-chain) in different FeLV proviruses. Although pathogenesis studies have still to be performed with these viruses, it is likely that both v-myc and v-tcr genes play leukaemogenic roles. It could be speculated that the v-tcr product provides a constitutive mitogenic signal from the cell membrane and the v-myc product cooperates in transformation at a later stage in the mitogenic signalling pathway in the nucleus.



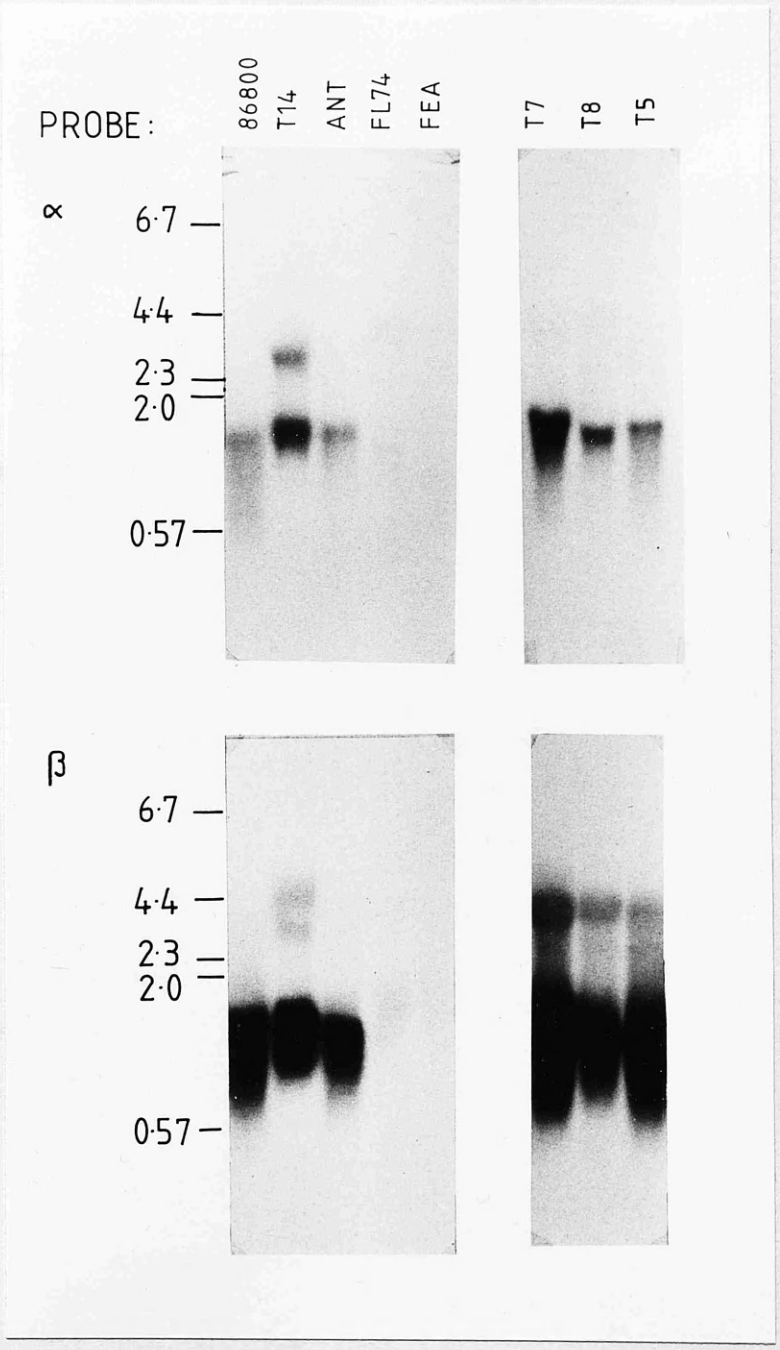


Fig.7.1. Northern blot analysis using TCR α- and β-chain probes of RNA from tumours and normal cells.

RNA samples were: tumours with a rearranged c-myc gene, T7, T8, T5; tumours with no detectable myc alteration, FL74, T14, 86800; normal cells, mitogen activated normal thymocytes, ANT, and embryo fibroblasts, FEA. After hybridisation, washes were performed in 2X SSC at 60°C (Chapter 2). Autoradiographs involved exposure overnight for β-chain results and over two nights for α-chain results.

Size markers were HindIII-digested λDNA labelled with ^{32}P.

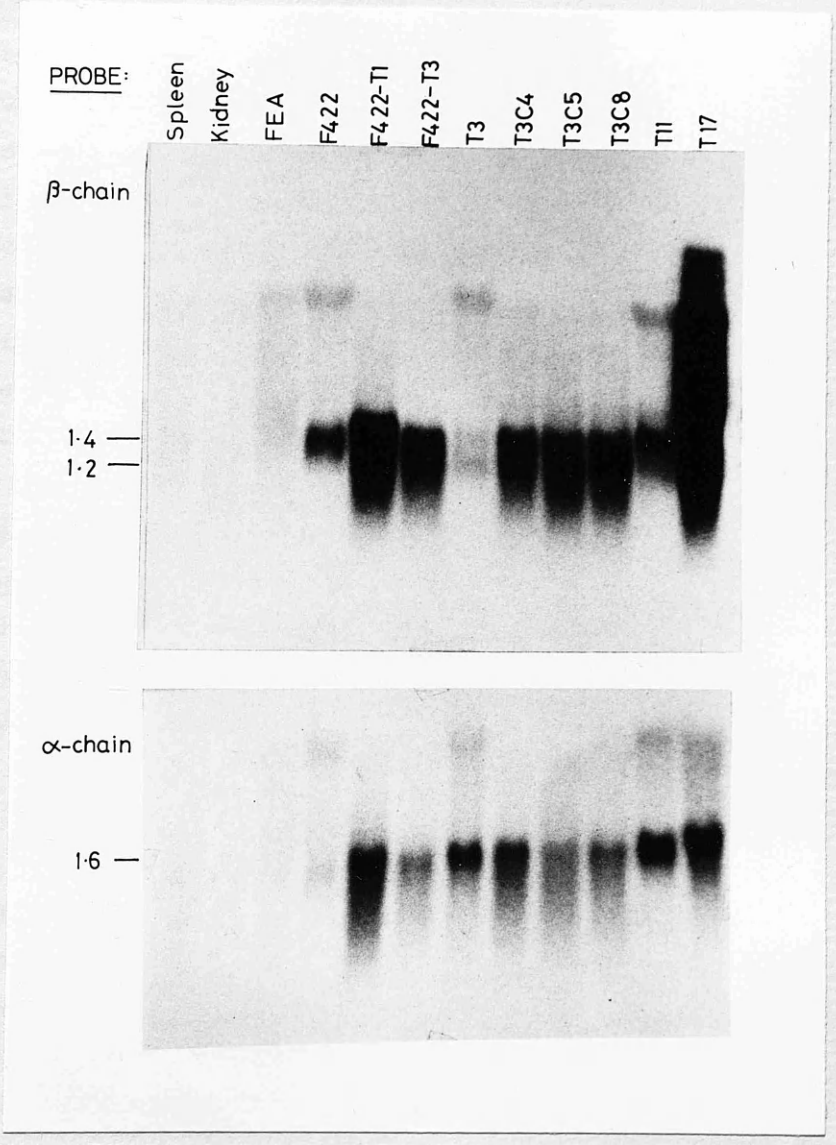


Fig.7.2. Northern blot analysis using TCR α- and β-chain probes of RNA from tumours containing an FeLV/myc virus.

RNA samples were from tumours described in Table 6.1, from normal spleen and kidney tissue and FEA embryo fibroblasts. Filter washing and autoradiography conditions and size markers were as described in Fig.7.2.

CHAPTER EIGHT

GENERAL SUMMARY AND DISCUSSION

In this study I have examined in detail the involvement of the myc gene in some feline lymphosarcomas, and have considered other genes which may play a part in this disease.

8.1 THE myc GENE IN FELINE LEUKAEMIAS

Unlike the situation in the murine and avian species where certain types of tumour are generally associated with one mode of retroviral activation of the myc gene, feline thymic lymphosarcomas show a variety of mechanisms. This diversity could result from study of naturally-occurring and experimental tumours in the cat whereas study of only experimentally-induced tumours in other species may cause bias in the findings. Experimental induction of tumours with retroviruses is known to be influenced by various factors including the host age and genetic status, the strain of virus, and the dose and route of virus inoculation (Teich et al, 1985). Thus, it is possible that experimental studies select for specific oncogenic mechanisms.

8.1.1 The feline c-myc gene (Chapter 3)

The feline c-myc gene is highly related to that of the other mammalian species (human, mouse), especially to that of man, but is less well related to that of the chicken. Homology is greatest across the coding exons 2 and 3, but is also considerable in the apparently non-coding exon 1 region of the the mammalian (feline, human, mouse) genes. The sequence of the three mammalian c-myc genes in each case reveals a putative dual promoter structure. The similarities in the mammalian genes were supported by S1 nuclease analyses showing that normal feline cells contain two major c-myc RNAs (P1 and P2) mapping to the two promoter-like elements, which possess a common 3' end corresponding to the most 3' of two possible polyadenylation signals (Chapter 5).

8.1.1 Transduction

Transduction of myc by FeLV is common in field case thymic tumours with seven independent isolates reported at present (Chapter 6). Each FeLV/myc virus is thought to have arisen de novo in the tumour in which it has been identified, since experimental evidence indicates that they are not transmitted horizontally (D. Onions et al, in press). This

proposal is also supported by sequence and S1 nuclease analysis of FeLV-myc recombination points, which have been found to differ between isolates, with an exception being a possible common 3', though not 5', terminus in the v-myc gene of the GT3 and GT11 viruses (Chapter 6).

Study of two FeLV/myc viruses shows them to induce thymic tumours which express TCR α and β-chain RNAs (Chapter 7; D. Onions et al, in press). This narrow oncogenic spectrum could be due to properties of FeLV and/or the myc gene. The enhanced transcriptional activity of FeLV LTRs in lymphoid cells (J. Casey, pers. comm.) may be an important factor and may account for the high levels of v-myc RNA present in tumours containing FeLV/myc viruses (Chapter 6).

Transduced myc genes in every case incorporate both coding exons 2 and 3 suggesting that an intact myc product without N or C-terminal truncation is necessary for oncogenic function (Chapter 3). In FeLV-CT4 and -FTT the predicted v-myc gene may not be fused to any viral structural gene product, but the LC isolate apparently codes for a gag-myc fusion product. The v-myc sequences of FeLV-CT4 and -FTT show few coding changes relative to the normal c-myc gene, and that of FeLV-LC shows none. However, the possible oncogenic role of structural alterations to the myc product remains to be systematically investigated, and this could be a useful extension to the studies described here.

8.1.2 Rearrangements of the c-myc locus

In addition to transduction, various forms of rearrangement of the c-myc locus have been found in thymic tumours (Chapter 4). One field and one experimental case in the Glasgow series of tumours and 8/11 experimental tumours studied by J. Mullins (pers. comm.) involve FeLV proviruses in the "enhancer insertion" mode upstream of the c-myc gene. Presumably some form of selection operates in these experimental tumours, which had all been induced by the highly pathogenic Rickard strain of FeLV, to favour this mode of myc alteration. Similarly, enhancer insertions are common in murine thymic lymphomas induced by highly leukaemogenic MuLVs (Selten et al, 1984). This may reflect possession of LTR transcriptional controls which are especially active in T-cells (Celander & Haseltine, 1984).

In contrast, tumour T24 appears to carry a truncated provirus in intron 1 in the "promoter insertion" mode, and may represent a

structural intermediate in the pathway to myc transduction by FeLV. The possible presence of a new FeLV/myc virus could be tested further for example, by investigating tumour formation following inoculation with cell-free extract from tumour T24.

Tumour T5 is another unusual case, since it seems to contain a single normal c-myc allele and two 3' rearranged alleles, which do not appear to involve FeLV sequences. It is intriguing to speculate that chromosomal rearrangements involving the c-myc and TCR gene loci may have occurred, as recently reported in a few human T-cell leukaemias (Shima et al, 1986; Erikson et al, 1986).

8.1.3 Expression of the c-myc gene in feline tumours

Analysis of RNA from the tumour series revealed different modes of c-myc expression. Rearrangements at the c-myc locus do not result in obvious abnormalities in transcription, since levels of c-myc transcripts are not grossly elevated, and RNA with altered structure is produced in only one case (tumour T24) (Chapter 5). However, the view that upstream FeLV insertions in tumours T7 and T8 contribute to c-myc transcriptional deregulation is supported by finding that these tumours contain a high ratio of P1 to P2 c-myc transcripts. This could result from enhancers in the FeLV LTRs preferentially activating the proximal (P1) promoter. But other factors must also be involved as some tumours with no apparent c-myc alteration display an equally high P1/P2 ratio. More conclusive interpretation of these studies awaits clearer definition of the normal pattern of c-myc transcription and of the cis and trans-acting factors which control its expression.

Evidence for the complexity of control of c-myc expression was uncovered in tumours expressing any of four different FeLV v-myc genes, where RNA could not be detected which derived from the ostensibly normal c-myc alleles in these tumours (Chapter 6). Similarly in tumour T24, RNA was detected which derived from the rearranged c-myc allele, but not the normal allele. Although it was not possible to distinguish between transcripts from the normal and rearranged c-myc alleles in tumours T7 and T8, precedent from other species suggests that the RNA may again derive from the rearranged alleles (ar-Rushdi et al, 1983; Bernard et al, 1983). Since this c-myc down-regulation was found consistently with different FeLV/myc viruses this may support models where c-myc expression is under negative autoregulatory control

(Leder et al, 1983), although the role of the myc product in this phenomenon remains to be examined. However, other explanations are not excluded; for example, c-myc normally may be down-regulated at a certain maturation stage in the target cells for transformation by FeLV/myc viruses.

My data for feline tumours, with those recorded for human and murine tumours, also suggest that loss of expression of normal c-myc alleles may prove to be a useful indicator of the presence of an oncogenically active myc gene, whether this is a rearranged c-myc or transduced v-myc sequence.

8.2 OTHER CELLULAR GENES INVOLVED IN FELINE LEUKAEMIAS

As the myc gene is only implicated in a subset of thymic lymphosarcomas, other genes may be involved in other cases. By analogy with MuLV-induced thymic lymphomas these genes may include the pim-1, tck or Mlvi loci (Cuypers et al, 1984; Voronova & Sefton, 1986; Tsichlis et al, 1983). An early part of my work involved screening the available tumour series for additional genetic rearrangements. This showed thymic tumour 84904 to carry a rearranged pim-1 restriction fragment which was also amplified <five-fold relative to the normal pim-1 fragment in the same tumour (Fig.8.1). It was not feasible to investigate linkage with FeLV sequences, as this tumour contained a very high copy number of proviruses, and the rearranged fragment would need to be cloned for further study.

A novel candidate gene for oncogenic function was a TCR β-chain gene transduced by FeLV in tumour T17 (Chapter 7). A molecular clone of this provirus is now available for functional analysis (R. Fulton et al, in press). Since the TCR normally is involved in transmitting mitogenic signals into the cell as well as in antigenic recognition (Smith, 1985) it is conceivable that the viral TCR (v-tcr) gene contributes to abnormal cell growth.

8.3 MULTISTAGE LEUKAEMOGENESIS

Several observations indicate that feline thymic lymphosarcomas arise by a series of events, consistent with theories of multistep oncogenesis (Klein & Klein, 1985). First, FeLV/myc viruses induce mono or oligoclonal tumours. If it is presumed that many cells may be infected by these viruses, this may be evidence that the cells which form tumours are selected by additional events. Recombinant MuLV/myc

constructs have also been found to induce clonal tumours in mice (Morse et al, 1986; Brightman et al, 1986). Secondly, FeLV/myc viruses have not been found to transform feline thymocytes, embryo fibroblasts or bone marrow cells in culture (D. Onions, pers. comm.) again suggesting the need for secondary events. This is supported by finding that co-transfection of an FeLV/myc virus and an EJ-ras gene transforms rat embryo cells, which is not achieved with either clone by itself (D. Doggett et al, submitted). Thirdly, experimental induction of clonal thymic tumours by FeLVs which do not carry an oncogene is generally a slow process (>1 year; Mackey et al, 1973; D. Onions, pers. comm.). The long latency may be explained as the time required for the occurrence of several oncogenic events in the same cell necessary for it to form a tumour.

A possible line of future investigation concerns the nature of such additional oncogenic events. One possibility is direct involvement of more than one oncogene. This may be the case in tumour T17 which in addition to an FeLV/tcr virus is known to contain an FeLV/myc virus (Chapter 7). This is the first report of two independent transductions of genes with oncogenic potential in the same tumour.

However, there are reports on tumours in other species where two cellular genes with oncogenic potential may sustain proviral insertions (Selten et al, 1984; Tsichlis et al, 1985; Peters et al, 1986). Analysis of murine thymic lymphomas suggested that emergence of a dominant tumour cell clone may be associated with additional proviral insertions, perhaps identifying genes for tumour progression (Cuypers et al, 1986). A variation on this theme may be feline tumour T5 which contains a c-myc rearrangement apparently not linked to FeLV sequences, and a low copy number of integrated FeLV elements (Chapter 4). Investigation of the cellular loci at which the FeLV sequences are inserted may identify genes with oncogenic potential which have cooperated with the rearranged c-myc gene in the genesis of this tumour.

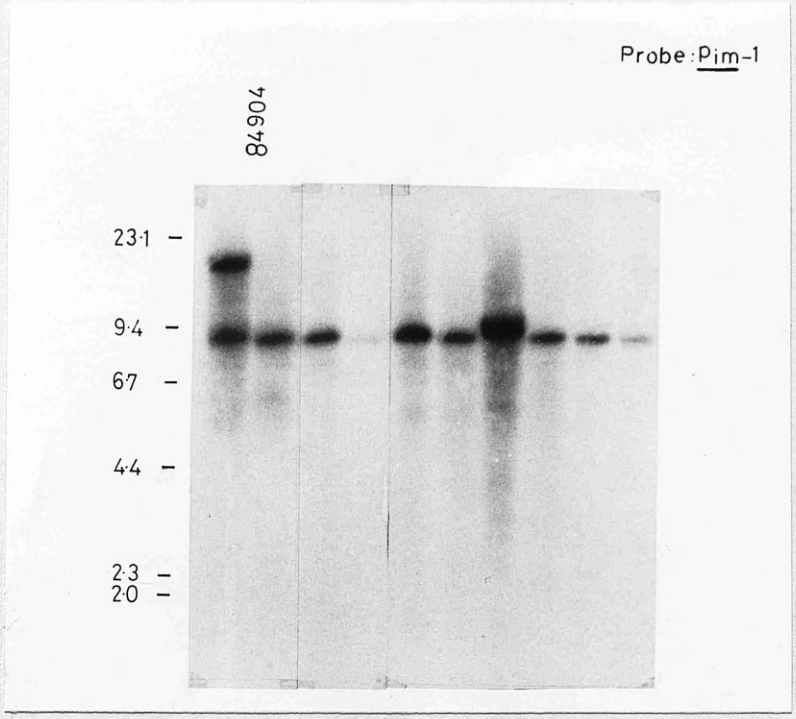


Fig.8.1. Southern blot analysis of tumours to screen for rearrangement of the pim-1 locus.

XbaI-digested DNAs were hybridised with a mouse Pim-1 plasmid insert (Table 2.1). Hybridisation was performed in 40% formamide at 35°C, and washes were in 2X SSC / 0.5% SDS at 39°C (Chapter 2).

No control normal tissue was available from the cat with tumour 84904. Controls where available were DNAs from normal kidney of the cats with tumours.

Lanes from left to right: tumours 84904, 84793, tumour T5 and control, tumour T10 and control, tumour 86503 and control, tumour 89407 and control.

Size markers were HindIII-digested λDNA, labelled with ^{32}P.

REFERENCES

Adams, J. M. (1985) Nature 315, 542-543.

Adams, J. M., Harris, A. W., Pinckert, C. A., Corcoran, L. M., Alexander, W. S., Cory, S., Palmiter, R. D. & Brinster, R. L. (1985) Nature 318, 533-538.

Alitalo, K., Schwab, M., Lin, C. C., Varmus, H. E. & Bishop, J. M. (1983) Proc. Nat. Acad. Sci. USA 80, 1707-1711.

Anderson, L. J., Jarrett, W. F. H., Jarrett, O. & Laird, H. M. (1971) J. Nat. Cancer Inst. 47, 807-817.

Ashley, D. J. B. (1969) Br. J. Cancer 23, 313-328.

ar-Rushdi, A., Nishikura, K., Erikson, J., Watt, R., Rovera, G. & Croce, C. M. (1983) Science 222, 390-393.

Barbacid, M. (1986) Trends Genet. 2, 188-192.

Battey, J., Moulding C., Taub, R., Murphy, W., Stewart, T., Potter, H., Lenoir, G. & Leder, P. (1983) Cell 34, 779-787.

Bentley, D. L. & Groudine, M. (1986a) Nature 321, 702-706.

Bentley, D. L. & Groudine, M. (1986b) Mol. Cell. Biol. 6, 3481-3489.

Benton, W. D. & Davis, R. W. (1977) Science 196, 180-182.

Bernard, O., Cory, S., Gerondakis, S., Webb E. & Adams, J. M. (1983) EMBO. J. 2, 2375-2383.

Besmer, P. (1983) Curr. Top. Microbiol. Immunol. 107, 1-27.

Besmer, P., Murphy, J. E., George P. C., Qiu, F-H., Bergold, P. J., Lederman, L., Snyder, H. W., Brodeur, D., Zuckerman, E. E. & Hardy W. D. (1986a) Nature 320, 415-421.

Besmer, P., Lader, E., George, P. C., Bergold, P. J., Qiu, F-H., Zuckerman, E. E. & Hardy, W. D. (1986b) J. Virol. 60, 194-203.

Bishop, J. M. (1983) Ann. Rev. Biochem. 52, 301-354.

Bishop, J. M. (1985) Cell 42, 23-38.

Bishop, J. M. & Varmus, H. E. (1985) pp. 249-356, in R. Weiss, N. Teich, H. Varmus & J. Coffin (eds.), RNA Tumour Viruses, 2nd edition, supplement, Cold Spring Harbor Laboratory , New York.

Bister, K. (1984) pp. 38-63, in J. M. Goldman & O. Jarrett (eds.), Mechanisms of Viral Leukaemogenesis, Churchill Livingstone, Edinburgh.

Braun, M. J., Deininger, P. L. & Casey, J. W. (1985) J. Virol. 55, 177-183.

Breathnach, R. & Chambon, P. (1981) Ann. Rev. Biochem. 50, 349-383.

Brightman, B. K., Pattengale, P. K. & Fan, H. (1986) J. Virol. 60, 68-81.

Brown, A. M. C., Wildin, R. S., Prendergast, T. J. & Varmus H. E. (1986) Cell 46, 1001-1009.

Bunte, T., Donner, P., Pfaff, E., Reis, B., Greiser-Wilke, I., Schaller, H. & Moelling, K. (1984) EMBO. J. 3, 1919-1924.

Campisi, J., Gray, H. E., Pardee, A. B., Dean, M. & Sonenshein, G. E. (1984) Cell 36, 241-247.

Care, A., Cianetti, L., Giampaolo, A., Sposi, N. M., Zappavigna, V., Mavilio, F., Alimena, G., Amadori, S., Mandelli, F. & Peschle, C. (1986) EMBO. J. 5, 905-911.

Casey, J. W., Roach, A., Mullins, J. I., Burck, K. B., Nicolson, M. O., Gardner, M. B. & Davidson, N. (1981) Proc. Nat. Acad. Sci. USA 12, 7778-7782.

Celander, D. & Haseltine, W. A. (1984) Nature 312, 159-162.

Chen, S. J., Holbrook, N. J., Mitchell, K. F., Vallone, C. A., Greengard, J. S., Crabtree, G. R. & Lin, Y. (1985) Proc. Nat. Acad. Sci. USA 82, 7284-7288.

Chen, I. S., Wachsman, W., Rosenblatt, J. D. & Cann, A. J. (1986) Cancer Surveys 5, 329-342.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) Biochem. 18, 5294-5299.

Cohen, J. C., Shank, P. R., Morris, V. L., Cardiff, R. & Varmus, H. E. (1979) Cell 16, 333-345.

Cohen, J. B., Unger, J., Rechavi, G., Canaani, E. & Givol, D. (1983) Nature 306, 797-799.

Collins, S. & Groudine, M. (1982) Nature 298, 679-681.

Collins, M. K. L. & Owen, M. J. (1985) Biochem. J. 230, 281-291.

Collins, MKL Tanigawa, G., Kissonerghis, A-M., Ritter, M., Price, K. M., Tonegawa, S. & Owen, M. J. (1985) Proc. Nat. Acad. Sci. USA 82, 4503-4507.

Corcoran, L. M., Adams, J. M., Dunn, A. R. & Cory, S. (1984) Cell 37, 113-122.

Cory, S., Graham, M., Webb, E., Corcoran, L. & Adams J. M. (1985) EMBO. J. 4, 675-681.

Crittenden, L. B. & Kung, H-J. (1984) pp. 64-88, in J. M. Goldman & O. Jarrett (eds.), Mechanisms of Viral Leukaemogenesis, Churchill Livingstone, Edinburgh.

Cuypers, H. T., Selten, G., Quint, W., Zijlstra, M., Maandag, E. R., Boelens, W., van Wezenbeek, P., Melief, C. & Berns, A. (1984) Cell 37, 141-150.

Cuypers, H. T. M., Selten, G. C., Zijlstra, M., De Goede, R. E., Melief, C. J. & Berns, A. (1986) J. Virol. 60, 230-241.

Dalla-Favera, R., Gelmann, E. P., Martinotti, S., Franchini, G., Papas, T. S., Gallo, R. C. & Wong-Staal, F. (1982) Proc. Nat. Acad. Sci. USA 79, 6497-6501.

Dani, C., Blanchard, J. M., Piechaczyk, M., Sabouty, S. E., Marty, L. & Jeanteur, P. (1984) Proc. Nat. Acad. Sci. USA 81, 7046-7050.

DePinho, R. A., Legouy, E., Feldman, L. B., Kohll, N. E., Yancopoulos, G. D. & Alt, F. W. (1986) Proc. Nat. Acad. Sci. USA 83, 1827-1831.

Dickson, C., Smith, R., Brookes, S. & Peters, G. (1984) Cell 37, 529-536.

Drabkin, H. A., Bradley, C., Hart, I., Bleskan, J., Li, F. P. & Patterson, D. (1985) Proc. Nat. Acad. Sci. USA 82, 6980-6984.

Dynan, W. S. & Tijan, R. (1985) Nature 316, 774-778.

Eisenman, R. N. & Thompson, C. B. (1986) Cancer Surveys 5, 309-327.

Enrietto, P. J., Payne, L. N. & Hayman, M. J. (1983) Cell 35, 369-379.

Enrietto, P. J. & Wyke, J. A. (1983) Adv. Cancer Res. 39, 269-314.

Erikson, J., ar-Rushdi, A., Drwinga, H. L., Nowell, P. C. & Croce, C. M. (1983) Proc. Nat. Acad. Sci. USA 80, 820-824.

Erikson, J., Finger, L., Sun, L., ar-Rushdi, A., Nishikura, K., Minowada, J., Finan, J., Emanuel, B. S., Nowell, P. C. & Croce, C. M. (1986) Science 232, 884-886.

Fahrlander, P. D., Sumegi, J., Yang, J-Q., Wiener, F., Marcu, K. B & Klein, G. (1985a) Proc. Nat. Acad. Sci. USA 82, 3746-3750.

Fahrlander, P. D., Piechaczyk, M. & Marcu, K. B. (1985b) EMBO. J. 4, 3195-3202.

Favoloro, J., Treisman, R. & Kamen, R. (1980) Meths. Enzymol. 65, 718-749.

Ficht, T. A., Chang, L-J. & Stoltzfus, C. M. (1984) Proc. Nat. Acad. Sci. USA 81, 362-366.

Fung, Y-K. T., Fadly, A. M., Crittenden, L. B. & Kung, H-J. (1981) Proc. Nat. Acad. Sci. USA 78, 3418-3422.

Fung, Y-K. T., Fadly, A. M., Crittenden, L. B. & Kung, H-J. (1982) Virol. 119, 411-421.

Fung, Y-K. T., Lewis, W. G., Crittenden, L. B. & Kung, H-J. (1983) Cell 33, 357-368.

Furley, A. J., Mizutani, S., Weilbaecher, K., Dhaliwal, H. S., Ford, A. M., Chan, L. C., Molgaard, H. V., Toyonaga, B., Mak, T., van den Elsen, P., Gold, D., Terhorst, C. & Greaves, M. (1986) Cell 46, 75-87.

Gazin, C., de Dinechin, S. D., Hampe, A., Masson, J-M., Martin, P., Stehelin, D. & Galibert, F. (1984) EMBO. J. 3, 383-387.

Gazin, C., Rigolet, M., Briand, J. P., Van Regenmortel, M. H. V. & Galibert, F. (1986) EMBO. J. 5, 2241-2250.

Gonda, T. J., Sheiness, D. & Bishop, J. M. (1982) Mol. Cell. Biol. 2, 617-624.

Graham, M., Adams, J. M. & Cory, S. (1985) Nature 314, 740-743

Greenberg, R., Hawley, R. & Marcu, K. B. (1985) Mol. Cell. Biol. 5, 3625-3628.

Greene, W. C., Leonard, W. J., Wano, Y., Svetlik, P. B., Peffer, N. J., Sodroski, J. G., Rosen, C. A., Goh, W. C. & Haseltine, W. A. (1986) Science 232, 877-880.

Hall, L. M. C. (1981) Ph.D. Thesis, Glasgow University.

Hanahan, D., (1983) J. Mol. Biol. 166, 557-580.

Hann, S. R., Thompson, C. B. & Eisenman, R. N. (1985) Nature 314, 366-369.

Hardy, W. D., Old, L. J., Hess, P. W., Essex, M. & Cotter, S. (1973) Nature 244, 266-269.

Hayflick, J., Seeburg, P. H., Ohlsson, R., Pfeiffer-Ohlsson, S., Watson, D., Papas, T. & Duesberg, P. H. (1985) Proc. Nat. Acad. Sci. USA 82, 2718-2722.

Hayman, M. (1983) Curr. Top. Microbiol. Immunol. 103, 109-125.

Hayward, W. S., Neel. B. G. & Astrin, S. M. (1981) Nature 290, 475-479.

Heaney, M. L., Pierce, J. & Parsons, J. T. (1986) J. Virol. 60, 167-176.

Heldin, C. H. & Westermark, B. (1984) Cell 37, 9-20.

Hollis, G. F., Mitchell, K. F., Battey, J., Potter, H., Taub, R., Lenoir, G. M. & Leder, P. (1984) Nature 307, 752-755.

Hood, L., Kroneberg, M. & Hunkapiller, T. (1985) Cell 40, 225-229.

Huang, C-C., Hay, N. & Bishop, J. M. (1985) Cell 44, 935-940.

Huebner, R. & Todaro, G. (1969) Proc. Nat. Acad. Sci. USA 64, 1087-1094.

Ihle, J. N. & Lee, J. C. (1982) Curr. Top. Microbiol. Immunol. 98, 85-101.

Jakobovits, E. B., Majors, J. E. & Varmus, H. E. (1984) Cell 38, 757-765.

Jarrett, O. (1984) pp. 135-154, in J. M. Goldman & O. Jarrett (eds.), Mechanisms of Viral Leukaemogenesis, Churchill Livingstone, Edinburgh.

Jarrett, O., Laird, H. M. &Hay, D. (1973) J. Gen. Virol. 20, 169-175.

Jarrett, O., Hardy, W. D., Golder, M. & Hay, D. (1978) Int. J. Cancer 21, 334-337.

Jarrett, O., Golder, M. C. & Stewart, M. F. (1982) Vet. Record 110, 225-228.

Jarrett, W. F. H., Martin, W. B., Crighton, G. W., Dalton, R. G. & Stewart, M. F. (1964) Nature 202, 566-568.

Kaczmarek, L., Hyland, J. K., Watt, R., Rosenberg, M. & Baserga, R. (1985) Science 228, 1317-1315.

Katzir, N., Rechavi, G., Cohen, J. B., Unger, T., Simoni, F., Segal, S., Cohen, D. & Givol, D. (1985) Proc. Nat. Acad. Sci. USA 82, 1054-1058.

Keath, E. J., Caimi, P. G. & Cole, M. D. (1984) Cell 39, 339-348.

Kelly, K., Cochran, B. H., Stiles, C. D. & Leder, P. (1983) Cell 35, 603-610.

Kingston, R. E., Baldwin, A. S. Jr. & Sharp, P. A. (1984) Nature 312, 280-282.

Klein, G. & Klein, E. (1985) Nature 315, 190-195.

Knudsen, A. G. (1971) Proc. Nat. Acad. Sci. USA 68, 820-823.

Lachman, H. M. & Skoultchi, A. I. (1984) Nature 310, 592-594.

Lang, J. C. & Spandidos, D. A. (1986) Anticancer Res. 6, 437-450.

Leder, P., Battey, J., Lenoir, G., Moulding, C., Murphy, W., Potter, H.,Stewart, T. & Taub, R. (1983) Science 222, 765-771.

Lee, W. F., Schwab, M., Westaway, D. & Varmus, H. E. (1985) Mol. Cell. Biol. 5, 3345-3356.

Levy, L. S., Gardner, M. B. & Casey, J. I. (1984) Nature 308, 853-856.

Levy, J. B., Iba, H. & Hanafusa, H. (1986) Proc. Nat. Acad. Sci. USA 83, 4228-4232.

Li, Y., Holland, C. A., Hartley, J. W. & Hopkins, N. (1984) Proc. Nat. Acad. Sci. USA 81, 6808-6811.

Linemeyer, D. L., Menke, J. G., Ruscetti, S. k., Evans, L. H. & Scolnick, E. M. (1982) J. Virol. 43, 223-233.

Linial, M. & Groudine, M. (1985) Proc. Nat. Acad. Sci. USA 82, 53-57.

Linial, M. Gunderson, N. & Groudine, M. (1985) Science 230, 1126-1132.

Little, C. D., Nau, M. M., Carney, D. N., Gazdar, A. F. & Minna, J. D. (1983) Nature 306, 194-196.

Lowy, D. R. & Willumsen, B. M. (1986) Cancer Surveys 5, 275-289.

Mackey, L. J., Jarrett, W. F. H., Jarrett, O. & Laird, H. M. (1972) J. Nat. Cancer Inst. 48, 16663-1670.

Makino, R., Hayashi, K. & Sugimura, T. (1984) 310, 697-698.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Laboratory Manual of Molecular Cloning, Cold Spring Harbor Publications, New York.

Mann, R., Mulligan, R. C. & Baltimore, D. (1983) Cell 33, 153-159.

Marshall, C. (1985) pp. 487-558, in R. Weiss, N. Teich, H. Varmus & J. Coffin (eds.), RNA Tumor Viruses, 2nd edition, supplement, Cold Spring Harbor Publications, New York.

Martin, G. S. (1970) Nature 227, 1021-1023.

Martin, P., Henry, C., Ferre, F., Duterque-Coquillaud, M., Lagrou, C., Ghysdael, J., Debuire, B., Stehelin, D. & Saule, S. (1986) EMBO. J. 5, 1529-1533.

Maxam, A. M. & Gilbert, W. (1980) Meths. Enzymol. 65, 499-560.

McGrath, M. S., Pillemer, E., Kooistra, D. A., Jacobs, S., Jerabek, L. & Weissman, I. L. (1980) Cold Spring Harbor Symp. Quan. Biol. 44, 1297-1304.

McLauchlan, J., Gaffney, D., Whitton, J. L. & Clements, J. B (1985) Nucleic Acids Res. 13, 1347-1368.

Morse, H. C., Hartley, J.w., Fredrickson, T. N., Yetter, R. A., Majumdar, C., Cleveland, J. L. & Rapp, U. R. (1986) Proc. Nat. Acad. Sci. USA 83, 6868-6872.

Mount, S. M. (1982) Nucleic Acids Res. 10, 459-472.

Mullins, J. I., Casey, J. W., Nicolson, M. O. & Davidson, N. (1980) Nucleic Acids Res. 8, 3287-3305.

Mullins, J. I., Brody, D. S., Binari, R. C. & Cotter, S. M. (1984) Nature 308, 856-858.

Murphree, A. L. & Benedict, W. F. (1984) Science 223, 1028-1033.

Murphy, W., Sarid, J., Taub, R., Vasicek, T., Battey, J., Lenoir, G. & Leder, P. (1986) Proc. Nat. Acad. Sci. USA 83, 2939-2943.

Naharro, G., Robbins, K.C. & Reddy, E. P. (1984) Science 223, 63-66.

Neel, B. G., Hayward, W. S., Robinson, H. L., Fang, J. & Astrin, S. M. (1981) Cell 23,323-334.

Neil, J. C., Hughes, D., MacFarlane, R., Wilkie, N. M., Onions, D. E., Lees, G. & Jarrett, O. (1984) Nature 308, 814-820.

Neil, J. C. & Onions, D. E. (1985) Anticancer Res. 5, 49-64.

Nilsen, T. W., Maroney, P. A., Goodwin, R. G., Rottman, F. M., Crittenden, L. B., Raines, M. A. & Kung, H-J. (1985) Cell 41, 719-726.

Noori-Daloii, M. R., Swift, R. A. & Kung, H-J. (1981) Nature 294, 574-576.

Nottenburg, C. & Varmus, H. E. (1986) Mol. Cell. Biol. 6, 2800-2806.

Nusse, R. (1986) Trends Genet. 2, 244-247.

Nusse, R. & Varmus, H. E. (1982) Cell 31, 99-109.

Nusse, R., van Ooyen, A., Cox, D., Fung, Y. K. T. & Varmus, H. E. (1984) 307, 131-136.

O'Brien, S. J. (1986) Trends Genet. 2, 137-142.

O'Donnell, P. V., Fleissner, E., Lonial, H., Koehne, C. F. & Reicin, A. (1985) J. Virol. 55, 500-503.

Onions, D. E., Jarrett, O., Testa, N., Frassoni, F. & Toth, S. (1982) Nature 296, 156-158.

Owen, F. L., Strauss, W. M., Murre, C., Duby, A. D., Hiai, H. & Seidman, J. G. (1986) Proc. Nat. Acad. Sci. USA 83, 7434-7437.

Papas, T. S. & Lautenberger, J. A. (1985) Nature 318, 237.

Parnes, J. R. (1986) Trends Genet. 2, 179-183.

Payne, G. S., Courtneidge, S. A., Crittenden, L. B., Fadly, A. M., Bishop, J. M. & Varmus, H. E. (1981) Cell 23, 311-322.

Payne, G. S., Bishop, J. M. & Varmus, H. E. (1982) Nature 295, 209-214.

Peters, G., Brookes, S., Smith, R. & Dickson, C. (1983) Cell 33, 369-377.

Peters, G., Lee, A. E. & Dickson, C. (1986) Nature, 320, 628-631.

Piechaczyk, M., Yang, J-Q., Blanchard, J-M., Jeanteur, P. & Marcu, K. B. (1985) Cell 42, 589-597.

Rabbitts, P. H., Forster, A., Stinson, M. A. & Rabbitts, T. H. (1985) EMBO. J. 4, 3727-3733.

Rabbitts, T. H., Forster, A., Hamlyn, P. & Baer, R. (1984) Nature 309, 592-597.

Raines, M. A., Lewis, W. G., Crittenden, L. B. & Kung, H-J. (1985) Proc. Nat. Acad. Sci. USA 82, 2287-2291.

Rapp, U. R., Cleveland, J. L., Brightman, K., Scott, A. & Ihle, J. N. (1985) Nature 317, 434-438.

Reddy, E. P., Reynolds, R. K., Watson, D. K., Schultz, R. A., Lautenberger, J. & Papas, T. S. (1983) Proc. Nat. Acad. Sci. USA 80, 2500-2504.

Reed, J. C., Nowell, P. C. & Hoover, R. G. (1985) Proc. Nat. Acad. Sci. USA 82, 4221-4224.

Reinherz, E. L., Kung, P. C., Goldstein, G., Levey, R. H. & Schlossman, S. F. (1980) Proc. Nat. Acad. Sci. USA 77, 1588-1592.

Reitsma, P. H., Rothberg, P. G., Astrin, S. M., Trial, J.,, Bar-Shavit, Z., Hall, A, Teitelbaum, S. L. & Kahn, A. J. (1983) Nature 306, 492-494.

Remmers, E. F., Yang, J-Q. & Marcu, K. B. (1986) EMBO. J. 5, 899-904.

Rickard, C. G., Post, J. E., Noronha, F. & Barr, L. (1969) J. Nat. Cancer Inst. 42, 987-1014.

Robinson, H. L. & Gagnon, G. C. (1986) J. Virol. 57, 28-36.

Rowley, J. D. (1984) Cancer Res. 44, 3159-3168.

Royer, H. D., Ramali, D., Acuto, O., Campen, T. J. & Reinherz, E. L. (1985) Proc. Nat. Acad. Sci. USA 82, 5510-5514.

Rubin, H. (1980) J. Nat. Cancer Inst. 64, 995-1000.

Ruddon, R. W. (1981) Cancer Biology, Oxford University Press, Oxford.

Saglio, G. Emanuel, B. S., Guerrasio, A. Giubellino, M. C., Serra, A., Lusso, P., Cambrin, G. R., Mazza, U., Malavasi, F., Pegoraro, L. & Foa, R. (1986) Cancer Res. 46, 1413-1417.

Saito, H., Hayday, A. C., Wiman, K., Hayward, W. S. & Tonegawa, S. (1983) Proc. Nat. Acad. Sci. USA 80, 7476-7480.

Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H. & Roe, B. (1980) J. Mol. Biol. 143, 161-178.

Selten, G., Cuypers, H. T., Zijlstra, M., Melief, C. & Berns, A. (1984) EMBO. J. 3, 3215-3222.

Selten, G., Cuypers, H. T. & Berns, A. (1985) EMBO. J. 4, 1793-1798.

Selten, G., Cuypers, H. T., Boelens, W., Robanus-Maandag, E., Verbeek, J., Domen, J., van Beveren, C. & Berns, A. (1986) Cell 46, 603-611.

Shen-Ong, G. L., Morse, H. C., Potter, M. & Mushinski, J. F. (1986) Mol. Cell. Biol. 6, 380-392.

Shibuya, M., Yokota, J. & Ueyama, Y. (1985) Mol. Cell. Biol. 5, 414-418.

Shih, C-K., Linial, M., Goodenow, M. M. & Hayward, W. S. (1984) Proc. Nat. Acad. Sci. USA 81, 4697-4701.

Shima, E. A., Le Beau, M. M., McKeithan, T. W., Minowada, J., Showe, L. C., Mak, T. K., Minden, M. D., Rowley, J. D. & Diaz, M. O. (1986) Proc. Nat. Acad. Sci. USA 83, 3439-3443.

Showe, L. C., Ballantine, M., Nishikura, K., Erikson, J., Kaji, H. & Croce, C. M. (1985) Mol. Cell. Biol. 5, 501-509.

Siebenlist, U., Hennighausen, L., Battey, J. & Leder, P. (1984) Cell 37, 381-391.

Slamon, D. J., deKernion, J. B., Verma, I. M. & Cline, M. J. (1984) Science 224, 256-262.

Smith, K. A. (1985) Cancer Cells 3, 205-210.

Soe, L. H. & Roy-Burman, P. (1984) Gene 31, 123-128.

Southern, E. (1975) J. Mol. Biol. 98, 503-517.

Stanton, L. W., Fahrlander, P. D., Tesser, P. M. & Marcu, K. B. (1984) Nature 310, 423-425.

Stanton, L. W., Schwab, M. & Bishop, J. M. (1986) Proc. Nat. Acad. Sci. USA 83, 1772-1776.

Steffen, D. (1984) Proc. Nat. Acad. Sci. USA 81, 2097-2101.

Stehelin, D., Varmus, H. E., Bishop, J. M. & Vogt, P. K. (1976) Nature 260, 170-173.

Stewart, M. A. (1983) Ph.D. Thesis, Glasgow University.

Stewart, M. A., Warnock, M., Wheeler, A., Wilkie, N. M., Mullins, J. I., Onions, D. E. & Neil, J. C. (1986a) J. Virol. 58, 825-834.

Stewart, M. A., Forrest, D., MacFarlane, R., Onions, D., Wilkie, N. M. & Neil, J. C. (1986b) Virol. 154, 121-134.

Stewart, T.A., Bellve, A.R. & Leder, P. (1984a) Science 226, 707-710.

Stewart, T. A., Pattengale, P. K. & Leder, P. (1984b) Cell 38, 627-637.

Stiles, C. D., Capone, G. T., Scher, C. D., Antoniades, H. N., Van Wyk, J. J. & Pledger, W. J. (1979) Proc. Nat. Acad. Sci. USA 76, 1279-1283.

Sumegi, J., Spira, J., Bazin, H., Szpirer, J., Levan, G. & Klein, G. (1983) Nature 306, 497-498.

Swanstrom, R., Parker, R. C., Varmus, H. E. & Bishop, J. M. (1983) Proc. Nat. Acad. Sci. USA 80, 2519-2523.

Swift, R. A., Shaller, E., Witter, R. L. & Kung, H-J. (1985) J. Virol. 54, 869-872.

Taub, R., Moulding, C., Battey, J., Murphy, W., Vasicek, T., Lenoir, G. & Leder, P. (1984a) Cell 36, 339-348.

Taub, R., Kelly. K., Battey, J., Latt, S., Lenoir, G., Tantrahavi, U., Tu, Z. & Leder, P. (1984b) Cell 37, 511-520.

Teich, N., Wyke, J. & Kaplan, P. (1985) pp. 187-248, in R. Weiss, N. Teich, H. Varmus & J. Coffin (eds.) RNA Tumor Viruses, 2nd edition, supplement, Cold Spring Harbor Laboratory, New York.

Temin, H. (1976) Science 192, 1075-1080.

Temin, H. (1980) Cell 21, 599-600.

Theilen, G. H., Kawakami, T. G., Rush, J. D. & Munn, R. J. (1969) Nature 222, 589-590.

Thompson, C. B., Challoner, P. B., Neiman, P. E. & Groudine, M. (1985) Nature 314, 363-366.

Toyoshima, K. & Vogt, P. K. (1969) Virol. 39, 930-931.

Trent, J., Meltzer, P., Rosenblum, M., Harsh, G., Kinzler, K., Mashal, R., Feinberg, A. & Vogelstein, B. (1986) Proc. Nat. Acad. Sci. USA 83, 470-473.

Tsichlis, P. N., Strauss, P. G. & Hu, L. F. (1983) Nature 302, 445-449.

Tsichlis, P. N., Strauss, P. G. & Lohse, M. A. (1985) J. Virol. 56, 258-267.

Van Beveren, C., Coffin, J., Hughes, S. (1985) pp.567-1148, in R. Weiss, N. Teich, H. Varmus & J. Coffin (eds.) RNA Tumor Viruses, 2nd edition, supplement, Cold Spring Harbor Laboratory, New York.

van Beneden, R. J., Watson, D. K., Chen, T. T., Lautenberger, J. A. & Papas, T. S. (1986) Proc. Nat. Acad. Sci. USA 83, 3698-3702.

van der Putten, H., Quint, W., van Raaj, J., Maandag, E. R., Verma, I. M. & Berns, A. (1979) Cell 24, 729-739.

Voronova, A. F. & Sefton, B. M. (1986) Nature 319, 682-685.

Waldman, T. A. (1986) Science 232, 727-732.

Walther, N., Jansen, H. W., Trachman, C. & Bister, K. (1986) Virol. 154, 219-223.

Watson, D. K., Reddy, E. P., Duesberg, P. H. & Papas, T. S. (1983) Proc. Nat. Acad. Sci. USA 80, 2146-2150.

Watt, R., Stanton, L. W., Marcu, K. B., Gallo, R. C., Croce, C. M. & Rovera, G. (1983a) Nature 303, 725-728.

Watt, R., Nishikura, K., Sorrentino, J., ar-Rushdi, A., Croce, C. M. & Rovera, G. (1983b) Proc. Nat. Acad. Sci. USA 80, 6307-6311.

Watt, R. A., Shatzman, A. R. & Rosenberg, M. (1985) Mol. Cell. Biol. 5, 448-456.

Weinberg, R. A. (1985) Science 230, 770-776.

Weiss, R., Teich, N., Varmus H. & Coffin, J. (1982) RNA Tumor Viruses, Cold Spring Harbor Laboratory, New York.

Westaway, D., Payne, G. & Varmus, H. E. (1984) Proc. Nat. Acad. Sci. USA 81, 843-847.

Westaway, D., Papkoff, J., Moscovici, C. & Varmus, H. E. (1986) EMBO. J. 5, 301-309.

Wiman, K., Clarkson, B., Hayday, A., Saito, H., Tonegawa, S. & Hayward, W. S. (1984) Proc. Nat. Acad. Sci. USA 81, 6798-6802.

Yang, J-Q., Bauer, S. R., Mushinski, J. F. & Marcu, K. B. (1985) EMBO. J. 4, 1441-1447.

Ymer, S., Tucker, W.Q.J., Sanderson, C.J., Hapel, A.J., Campbell, H.D. & Young, I.G. (1985) Nature 317, 255-258.

Yoshikai, Y., Anatoniou, A., Clark, S. P., Yanagi, Y., Sanster, R., Elsen, P., Terhorst, C. & Mak, T. N. (1984) Nature 312, 521-524.