A TRANSGENIC MODEL TO STUDY THE ROLE OF ONCOGENES AND TUMOUR SUPPRESSOR GENES IN T CELL LYMPHOMA

by

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SUMMARY

Transgenic mice have previously been used as an effective *in vivo* model to examine the role of genetic lesions in tumourigenesis. The work presented in this thesis describes a transgenic approach designed to investigate the consequences of deregulated c-*myc* and loss of the p53 tumour suppressor gene in the pathogenesis of T-cell lymphoma.

Transgenic mice positive for both a CD2-*myc* transgene and homozygous p53 null mutation were generated by crossing these parental strains together. The $p53^{-/-}$/CD2-myc^{+} transgenic mice developed highly malignant, clonal T-cell lymphomas with increased frequency and reduced latency compared to either genotype alone. Tumours were detected in young $p53^{-/-}$/CD2-myc^{+} mice as early as 27 days of age with clinical manifestation of disease occurring at between 50 and 86 days of age. These studies revealed the potent synergy between deregulated c-*myc* and p53 loss in T-cell lymphomagenesis. The latency and clonality of $p53^{-/-}$/CD2-myc^{+} tumours however, suggested that CD2-*myc* and $p53^{-/-}$ lesions alone were insufficient for the induction of a malignant phenotype. This interpretation was confirmed by infection of $p53^{-/-}$/CD2-myc^{+} neonatal mice with Moloney murine leukaemia virus (MoMuLV) which resulted in a further acceleration of tumour onset. The mechanism of synergy between CD2-*myc* and $p53^{-/-}$ is unclear but gross examination of malignant tumours demonstrated that the levels of apoptosis in $p53^{-/-}$/CD2-myc^{+} lymphomas were not significantly different to parental groups. This suggests that p53-dependent, c-*myc* mediated apoptosis does not appear to play a major role at this stage of tumour development.

Tumourigenesis was not accelerated in animals heterozygous for the p53 null mutation suggesting that loss of both wild type p53 alleles was required for efficient synergy with the CD2-*myc* transgene. However studies on $p53^{+/-}$/CD2-myc^{+} mice implied that loss of the remaining allele could confer a selective advantage on c-*myc* expressing tumour cells.

Loss of the p53 tumour suppressor gene also collaborated with MoMuLV infection in the development of murine T-cell tumours. However the relatively weak synergy between these lesions compared to that observed with p53 loss and deregulated c-*myc* may be suggestive of a functional overlap between MoMuLV and p53 loss.

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DECLARATION

I hereby declare that the work presented in this thesis is original and was conducted by the author under supervision except where stated.

I certify that no part of this thesis has been submitted previously for the award of a degree to any University but has been reproduced in parts in the following scientific papers:-

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ABBREVIATIONS

ALV	avian leukosis virus
APC	adenomatous polyposis coli
bHLH-LZ	basic region helix-loop-helix leucine zipper
bp	base pair
BSA	bovine serum albumin
CD	cluster designation
cdk(s)	cyclin-dependent kinase(s)
cDNA	complementary DNA
CIP1	cdk-interacting protein
cm	centimeter
CSF-1	colony stimulating factor 1
DCC	deleted in colorectal cancer
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
Eμ	immunoglobulin heavy chain enhancer
ES cell	embryonic stem cell
F-MuLV	Friend murine leukaemia virus
FAP	familial adenomatous polyposis
FITC	fluorescein isothiocyanate
g	gram
GADD	growth arrest and DNA damage-inducible
GAPDH	glyceraldehyde phosphate dehydrogenase
HCL	hydrogen chloride
HNPCC	hereditary non-polyposis colorectal cancer
HPV	human papillomavirus
HSV	herpes simplex virus

ICE	interleukin-1β converting enzyme
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
Il	interleukin
kb	kilobase
KCl	potassium chloride
kDa	kilodalton
l	litre
LFS	Li-Fraumeni syndrome
M	molar
MDM2	murine double minute 2
μg	microgram
mg	milligram
$MgCl_2$	magnesium chloride
μl	microlitre
ml	millilitre
μm	micrometer
μM	micromolar
mm	millimeter
mM	millimolar
MMTV	mouse mammary tumour virus
MoMuLV	Moloney murine leukaemia virus
MOPS	(3-[N-morpholino] propane-sulfonic acid)
mRNA	messenger ribonucleic acid
MTS1	multiple tumour suppressor gene 1
NaCl	sodium chloride
NF1	neurofibromatosis type 1
NF2	neurofibromatosis type 2
ng	nanogram
nm	nanometre
ODC	ornithine decarboxylase
$p53^+$	p53 wild type positive

$p53^{-}$	p53 wild type negative
$p53^{-/-}$	homozygous for the p53 null mutation
$p53^{+/-}$	heterozygous for the p53 null mutation
$p53^{+/+}$	wild type p53
$p53^{-/-}$/CD2-*myc*$^{+}$	homozygous p53 null / CD2-*myc* transgene positive
$p53^{+/-}$/CD2-*myc*$^{+}$	heterozygous p53 null / CD2-*myc* transgene positive
$p53^{+/+}$/CD2-*myc*$^{+}$	wild type p53 / CD2-*myc* transgene positive
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
r.p.m.	revolutions per minute
RB (pRb)	retinoblastoma tumour suppressor gene (protein)
RNA	ribonucleic acid
RSV	Rous sarcoma virus
SDS	sodium dodecyl sulphate
SV40	simian virus 40
TBP	TATA- binding protein
TcR	T-cell receptor
TdT	terminal deoxynucleotidyl transferase
TNF	tumour necrosis factor
TUNEL	TdT-mediated dUTP-biotin nick end labelling
UV	ultra violet
V	volts
WAF1	wild type p53-activated fragment 1
WT1	Wilms' tumour gene

CHAPTER 1.

GENERAL INTRODUCTION.

1.1 CANCER AND GENETICS.

The general acceptance that cancer is a disease of malfunctioning genes has followed many years of intensive research into the underlying causes of tumour development. Neoplastic cancer cells are derived from cells which have escaped the normal homeostatic mechanisms of physiological growth. Unrestrained growth of cancer cells is a consequence of alterations in the expression or biochemical function of genes, caused by genetic damage. The discovery that a large number of genes which are mutated in cancer are involved in cellular growth has led to a greater understanding of the mechanisms governing normal growth as well as neoplastic processes. Early evidence for the existence of cancer-causing genes came from studies of RNA tumour viruses and the finding that sequences of the viral genome were responsible for the induction of tumours. The identification of DNA anomalies and isolation of cellular genes associated with spontaneous tumour development further emphasized that genetic damage was intrinsic to the pathogenesis of tumours. DNA alterations found in spontaneous tumours include point mutations, deletions, chromosomal rearrangements and gene amplification (Bishop, 1991). Many causative agents of cancer have now been identified, for example radiation, tobacco and certain chronic infections (reviewed by Ames *et al.*, 1995). At least some of these agents are known to act directly as DNA mutagens.

Genetic mutations can result in the activation/mutation of dominant oncogenes, or the inactivation of tumour suppressor genes. Dominant oncogenes are derived from cellular genes, collectively known as proto-oncogenes, which are involved in normal cell growth and differentiation. Inappropriate expression of these genes or their gene products leads to a gain of function with increased biological activity. In contrast, tumour associated damage to tumour suppressor genes results in loss of normal function. The gene products of this class of genes generally restrict cell growth and therefore it is the absence of these gene products which contribute to neoplasia. Tumour suppressor gene

mutations are generally regarded as being recessive as both copies of the gene must be inactivated for functional loss of the gene product (Knudson, 1985).

Most malignancies occur as a result of both dominant and recessive lesions. Indeed neoplastic transformation is a multistep program which requires numerous changes in the biochemical function of cells (Klein and Klein, 1985). Consequently, damage to multiple genes must occur for the initiation and progression of a single tumour (Weinberg, 1989; Hunter, 1991). One of the best characterised models of multistep tumourigenesis is colorectal cancer. Changes in at least four or five defined genes are required for the development of a malignant colorectal tumour including both mutational activation of oncogenes and mutational inactivation of tumour suppressor genes (Fearon and Vogelstein, 1990). It has been suggested that it is the accumulation of genetic changes rather than the order in which they take place which is important for the development of a malignant tumour (Fearon and Vogelstein, 1990). The accretion of multiple genetic lesions has also been observed in various other tumour types (Bishop, 1991). Evidence for the multistep nature of carcinogenesis has additionally been provided from studies of transgenic mice which require more than one genetic abnormality for the development of tumours (see section 1.7; Hanahan, 1988). Although co-operation between a number of genetic lesions has been recognised, the mechanism of how these events combine to achieve the malignant phenotype is still largely unclear (Hunter, 1991).

1.2 APOPTOSIS.

Normal cell homeostasis is a balance between cell proliferation and cell death. Neoplasia results from a disruption to this equilibrium, when the rate of cellular proliferation exceeds that of cell death. While deregulation of cellular proliferation has long been associated with tumour development, only in recent years has the importance of cell death in tumourigenesis been widely acknowledged (Marx, 1993). It has been shown that the function of certain oncogenes and tumour suppressor genes is to regulate the cell death process (reviewed by Williams and Smith, 1993; Hoffman and Liebermann, 1994). Inhibition of cell death through the deregulation of these genes can result in aberrant cell

survival and contribute to tumourigenesis. Furthermore, genetic co-operation between lesions which result in enhanced proliferation and increased cell survival appear to be a potent combination for tumourigenesis (McDonnell, 1993a).

Apoptosis is an innate mode of cell death which is an essential part of many cellular processes, including embryonic development, tissue homeostasis and immune regulation (Arends and Wyllie, 1991). An active form of death, apoptosis is characterised by several morphological and biochemical features including nuclear condensation, increased cell density, degradation of DNA, cell surface blebbing and cell fragmentation (Arends *et al.*, 1990; Arends and Wyllie, 1991; Cohen, 1993; Wyllie, 1993). The apoptotic cell splits into membrane-bound fragments of various sizes called apoptotic bodies. Apoptotic cells are phagocytosed by neighbouring cells or macrophages and therefore do not induce an inflammatory reaction (Savill *et al.*, 1990; Savill *et al.*, 1993).

The process of apoptosis appears to be a complex mechanism which involves several different signals, pathways and effectors. Considerable progress has been made in defining the pathways which lead to the execution of cell death (reviewed by Oltvai and Korsmeyer, 1994; Green and Martin, 1995). Induction of apoptosis can arise from physical insults in the form of genetic damage (e.g. by ionizing radiation), ligation of Fas and tumour necrosis factor (TNF) cell surface receptors, glucocorticoid hormones, cytokine deprivation and various other environmental stimuli (Zheng *et al.*, 1995; Nagata and Golstein, 1995; Compton and Cidlowski, 1992; Thompson, 1995). Some inducers of apoptosis have been found to activate signal transduction pathways like that of the sphingomyelin pathway resulting in increased ceramide accumulation (Jarvis *et al.*, 1994). Following induction, apoptosis is regulated by mediator genes, several of which have been identified (Williams and Smith, 1993; Hoffman and Liebermann, 1994). These genes are often found to be altered and deregulated in tumourigenesis resulting in apoptotic inhibition and inappropriate cell survival. It is the regulatory genes which are presumed to influence the final execution pathways that activate the cellular changes characteristic of apoptotic cell death. The execution of the final stages in the apoptotic pathway probably occurs through members of the interleukin-1β converting enzyme

(ICE) family of cysteine proteases (Whyte and Evan, 1995; Nicholson *et al.*, 1995; Martin and Green, 1995).

1.3 ONCOGENES.

Proto-oncogenes are cellular genes which are important for cellular proliferation. Oncogenes are the mutant alleles of proto-oncogenes, many of which promote deregulated proliferation in neoplastic cells. The identification of oncogenes has come from several lines of investigation (reviewed by Varmus, 1984; Bishop, 1987; Spandidos and Anderson, 1989) with approximately one hundred oncogenes having been described by 1994 (Hesketh, 1994a).

1.3.1 The Identification of Oncogenes.

The first oncogenes to be identified were found within the genomes of acute transforming retroviruses (reviewed by Varmus, 1984), the Rous sarcoma virus (RSV) being the classical example. First observed by Rous in 1911 to induce avian sarcomas, RSVs transforming abilities were found to be dependent on the presence of a single gene *src* (Bishop, 1985). Further investigation of RSV and other acutely transforming retroviruses revealed that each of the viral oncogenes had a cellular homologue (Varmus, 1984). Retroviral oncogenes arise by transduction of cellular genes to the viral genome during the replicative life cycle of the virus when viral and host genomes recombine. Transduction of cellular genes places them under the regulation of viral promoters leading to deregulated expression. Viral oncogenes differ from their cellular homologues as they do not contain introns and are often altered (Onions, 1991). Oncogenes identified as transduced genes in acute transforming retroviruses include c-*myc*, c-*myb*, c-Ha-*ras*, c-Ki-*ras*, c-*erbB*, c-*mos*, c-*abl*, c-*raf*, c-*fos* and c-*sis* (Varmus, 1984; Onions and Jarrett, 1987).

Integration of retroviruses into cellular DNA can itself be a mutagenic event. Slow transforming retroviruses, which do not express viral oncogenes, activate cellular genes

by insertional mutagenesis (Kung *et al.*, 1991). In this process, the retrovirus damages the cellular gene directly or places the gene under viral regulatory controls (Kung *et al.*, 1991; Tsichlis and Lazo, 1991). Viral insertion takes place randomly throughout the host DNA. However cells with insertions near to, or at particular proto-oncogenes acquire a selective growth advantage (Kung *et al.* 1991). Many oncogenes have been detected at common viral integration sites including c-*myc*, c-*myb* and c-*erbB* (Tsichlis and Lazo, 1991; van Lohuizen and Berns, 1990). Targets of insertional mutagenesis have also led to the identification of several novel oncogenes. The *pim*-1 gene for example was discovered in mouse T-cell lymphomas induced by the murine leukaemia virus MoMuLV (Cuypers *et al.*, 1984). The *int*-1, *int*-2, *ahi*-1, *evi*-1, *evi*-2, *mlvi*-1, *mlvi*-2, and *mlvi*-3 genes have similarly been identified as proviral target genes (Onions and Jarrett, 1987; Tsichlis and Lazo, 1991; Hesketh, 1994a).

DNA tumour viruses also have oncogenic potential but unlike RNA tumour viruses do not contain transduced cellular genes, nor activate host oncogenes upon viral infection. DNA tumour viruses express viral genes which generally do not have cellular homologues but are capable of transformation (Bishop, 1985). The transforming properties of several of these genes including simian virus 40 (SV40) large T antigen, adenovirus E1A and E1B and papilloma virus E6 and E7 genes are related to their ability to complex with cellular proteins as will be discussed in later sections.

Cellular oncogenes have been detected in spontaneous tumours at chromosomal translocations (Varmus, 1984; Haluska *et al.*, 1987). Chromosomal translocations result in portions of two chromosomes becoming joined together. Proto-oncogenes situated at the breakpoint region between these chromosomes can be activated as a consequence of the translocation (Haluska *et al.*, 1987). The c-*myc* and c-*abl* oncogenes are two genes which have been identified at chromosomal translocations (Spandidos and Anderson, 1989). The *bcl*-2 gene was identified as a new oncogene from its location at a chromosomal translocation breakpoint which placed it under the regulation of the immunoglobulin heavy-chain locus (Bakhshi *et al.*, 1985).

DNA amplification in a number of tumour types has also led to the identification of cellular oncogenes. Amplification of proto-oncogenes results in high levels of the protein product. The c-*myc*, c-*erbB*, c-*abl*, c-*myb* and c-Ki-*ras* oncogenes have been detected in amplified DNA (Varmus, 1984). Novel genes identified from amplified DNA include the N-*myc* and L-*myc* oncogenes (Schwab *et al.*, 1983; Nau *et al.*, 1985).

1.3.2 The Functions of Proto-oncogenes and Oncogenes.

The subcellular location and properties of numerous proto-oncogenes have been determined (reviewed by Varmus and Bishop, 1986; Spandidos and Anderson, 1989; Hunter, 1991; Hesketh, 1994a). Proto-oncogenes are located at the plasma membrane, in the cytoplasm and in the nucleus. These genes appear to have diverse functions at all levels of the cell signalling pathway from secreted growth factors and membrane receptors to protein kinases and transcription factors (Jones and Kane, 1996).

Some proto-oncogenes are related to growth factors, the *sis* oncogene for example encodes a homologue of the platelet-derived growth factor, PDGF (Waterfield *et al.*, 1983). Constitutive expression of growth factors will provide sustained activation of cell proliferation. This same effect could be achieved by deregulating cell surface growth factor receptors. Indeed, a variety of oncogenes encode cell surface growth factor receptors such as the truncated epidermal growth factor (EGF) receptor and the colony stimulating factor 1 (CSF-1) receptor encoded by the c-*erbB* and c-*fms* oncogenes respectively. Oncogenes which encode cell surface growth factor receptors belong to the tyrosine kinase and serpentine receptor families (Hunter, 1991). Several proto-oncogenes, located at the membrane and in the cytoplasm, are involved in mediating receptor signalling. Proteins encoded by these genes are protein-tyrosine kinases (for example encoded by c-*src*), protein-serine/threonine kinases (for example encoded by c-*raf*) and the membrane-associated G proteins (encoded by the *ras* family of oncogenes). Proteins encoded by the oncogenes c-*fos*, c-*jun*, c-*myc* and others belong to a class of nuclear transcription factors (Eisenman, 1989). These oncoproteins are effectors of the signalling pathways and control the transcription of genes involved in the induction of

cell cycle progression. Point mutations or deletions of these oncogenes can result in their constitutive expression leading to continuous cell cycling and uncontrolled growth.

Co-operation between oncogenes occurs between those genes which control different signalling pathways (Hunter, 1991). It is possible however that combinations of certain oncogenes controlling the same pathway would be activated to strengthen a particular pathway. Synergy often occurs between cytoplasmic and nuclear oncogenes as exemplified by the co-operation between the *ras* and *myc* oncogenes (Land *et al.*, 1983). Deregulation of a cytoplasmic oncogene may induce a pathway leading to activation of a second nuclear oncogene i.e. c-*fos*, which acts on a different set of target genes (Hunter, 1991).

Oncogenic co-operation can exist between two nuclear transcription factors. This is observed with the c-*fos* and c-*jun* oncogenes (Gillespie, 1991). These genes are constitutively activated by gene deletions which result in loss of normal regulatory elements (Hesketh, 1994a). The gene products of c-*jun* and c-*fos* form stable heterodimers which enhance the DNA binding affinity of the c-*jun* protein to the transcription factor AP-1 in order to induce gene transcription (Eisenman, 1989; Gillespie, 1991). Constitutive activation of both of these genes may result in inappropriate gene expression and uncontrolled proliferation.

The *ras* family of oncogenes are implicated in a large number of cancers including carcinomas, sarcomas and leukaemias indicating that mutation of *ras* is a potent tumourigenic event. The highly conserved *ras* family includes the H-*ras*, K-*ras* and N-*ras* genes, isolated from Harvey murine sarcoma virus, Kirsten murine sarcoma virus and spontaneous tumours respectively (Hesketh, 1994b). These genes encode membrane bound G proteins which bind guanine nucleotides and have GTPase activity. The *ras* gene products are signal transducers for cell surface growth factor receptors becoming active during growth factor stimulation (Hunter, 1991). Single amino acid substitutions constitutively activate the *ras* gene (Varmus, 1984). Activated *ras* is thought to co-operate with tyrosine kinases to cause transformation. It is possible that frequent mutation of the *ras* oncogene in tumours arises because of the involvement of the gene in

a large number of signalling pathways. Therefore activation of the *ras* oncogene would be the equivalent of two or three oncogenes acting as growth factors or growth factor receptors.

1.3.3 The *bcl*-2 Oncogene.

The *bcl*-2 oncogene, identified at the t(14;18) translocation breakpoint in follicular lymphoma (Bakhshi *et al.*, 1985), differs from other dominant oncogenes as it does not regulate cellular proliferation but rather regulates cell survival through the repression of apoptotic cell death (Vaux *et al.*, 1988; Hockenbery *et al.*, 1990). The *bcl*-2 gene inhibits apoptosis in response to a number of different stimuli (Sentman *et al.*, 1991) however it is still not clear how this effect is carried out. A family of Bcl-2-like proteins have now been identified including three viral genes (Hacker and Vaux, 1995). Like Bcl-2, some of the Bcl-2 family proteins inhibit apoptosis but others, for example the Bax protein, have been found to induce cell death (Vaux, 1993; Hacker and Vaux, 1995). It is probable that the Bcl-2 family proteins act as a network to regulate apoptosis. Indeed it is thought that Bcl-2 must heterodimerise with Bax to suppress cell death. Bax homodimers induce cell death, therefore Bcl-2 may protect cells from dying by binding to Bax (Oltvai and Korsmeyer, 1994; Hacker and Vaux, 1995). However as other proteins have been found to bind both Bcl-2 and Bax, it is likely that regulation of apoptosis by the *bcl*-2 family is a complex process.

The *bcl*-2 gene product is a 25 kilodalton integral mitochondrial membrane protein (Hockenbery *et al.*, 1990). Consistent with the role of *bcl*-2 in repressing cell death, the protein has been found to be restricted to long-lived cell types (Hockenbery *et al.*, 1991; Cory, 1995). The *bcl*-2 gene is found to be expressed in a number of tissue types (Hockenbery *et al.*, 1991) and while expression is not essential for embryonic development, it is required to maintain normal homeostasis in adult tissues (Veis *et al.*, 1993). Deregulation of the *bcl*-2 gene leads to reduced apoptosis and the accumulation of non-dividing cells (Vaux *et al.*, 1988; McDonnell *et al.*, 1989; McDonnell *et al.*, 1990). Overexpression of *bcl*-2 alone is not a potent oncogenic signal, however it can effectively synergise with other oncogenic lesions, particularly overexpression of the c-

myc oncogene (Vaux *et al.*, 1988; Strasser *et al.*, 1990; McDonnell and Korsmeyer, 1991). It is possible that by prolonging cell survival, *bcl*-2 contributes to tumourigenesis by increasing the cell pool vulnerable to further oncogenic changes.

1.4 THE C-*MYC* ONCOGENE.

1.4.1 The *myc* Oncogene Family.

The v-*myc* oncogene was first discovered as the transforming sequence in avian myelocytoma virus MC29 (Duesberg *et al.*, 1977). The c-*myc* gene was subsequently isolated and shown to be the cellular homologue of the viral v-*myc* gene (Vennstrom *et al.*, 1982). Deregulation of the c-*myc* proto-oncogene has been frequently associated with a large number of diverse neoplasms and has been implicated as having an important role in normal cellular growth control (reviewed by Cole, 1986; Marcu *et al.*, 1992). The highly conserved c-*myc* gene belongs to a family of *myc*-related genes of which the best characterised are N-*myc* and L-*myc*. The N- and L-*myc* genes, which share limited regions of homology with c-*myc*, were identified by their overexpression in neuroblastoma and small cell lung cancer respectively (Schwab *et al.*, 1983; Nau *et al.*, 1985).

1.4.2 The c-*myc* Gene.

The c-*myc* gene, like N- and L-*myc*, has a three exon structure. The first exon has been shown to be a long non-coding sequence with important regulatory roles (Spencer and Groudine, 1991). Exons 2 and 3 contain the major coding regions. A high degree of amino acid homology exists between species especially in the coding regions where, for example, the mouse and human genes share over 90% identity (Bernard *et al.*, 1983). Transcriptional initiation occurs at two major promoter sites, P1 and P2, and gives rise to c-*myc* mRNA species of approximately 2.4 kb and 2.2 kb respectively (Battey *et al.*, 1983; Bernard *et al.*, 1983). P2 is generally found to be the predominant promoter site generating 75-90% of c-*myc* mRNAs (Stewart *et al.*, 1984b; Spencer *et al.*, 1990).

Expression of the c-*myc* gene occurs in normal growing cells, with 10- to 40-fold higher mRNA levels in proliferating cells than in quiescent cells (Kelly *et al.*, 1983; Keath *et al.*, 1984). Following mitogenic stimulation, c-*myc* RNA is rapidly induced in the G1 phase of the cell cycle suggesting involvement of c-*myc* in the transition from quiescence to proliferation (Kelly *et al.*, 1983). Following the initial transient induction, constant levels of c-*myc* RNA are continuously expressed during proliferation in a cell cycle independent manner (Thompson *et al.*, 1985; Hann *et al.*, 1985). Further evidence that c-*myc* expression was required for cellular growth was provided when transfection of anti-c-*myc* antibodies in HL60 cells resulted in specific growth inhibition (Iguchi-Ariga *et al.*, 1987). Moreover, high levels of c-*myc* expression correlated with rapidly proliferating cells in the late stages of embryonic development (Schmid *et al.*, 1989). The requirement for c-*myc* expression during embryonic development has since been demonstrated by the embryonic lethality which occurs in mice homozygous for a c-*myc* null mutation (Davis *et al.*, 1993).

It has been observed that levels of c-*myc* expression decrease during cellular differentiation in mouse erythroleukaemia cells (Lachman and Skoultchi, 1984). Similar observations have been recorded in a number of cell types suggesting that down-regulation of c-*myc* may be necessary for some cell types to exit cell cycle and undergo terminal differentiation (reviewed by Spencer and Groudine, 1991; Marcu *et al.*, 1992). Further support for the hypothesis that high levels of c-*myc* expression maintain cells in an undifferentiated form has been provided by transgenic studies. In animals overexpressing a c-*myc* transgene large populations of proliferating cells accumulated which did not proceed through the expected differentiation pathway (Langdon *et al.*, 1986).

It would appear that c-*myc* expression is controlled by a complex set of regulatory mechanisms operating at various transcriptional and post-transcriptional levels (Spencer and Groudine, 1991; Marcu *et al.*, 1992). Post-transcriptional regulation of c-*myc* mRNA was found to be important for the transition from growth arrest to proliferation in a study by Blanchard *et al.* (1985). A role for exon 1 in regulating the stability of mRNA

has been proposed (Rabbitts *et al.*, 1985). Another model, not mutually exclusive, is that c-*myc* autosuppression may play a role in the control of c-*myc* expression at the level of transcriptional initiation (Penn *et al.*, 1990a). In Burkitt's lymphoma cells only the translocated allele was expressed with no detectable expression of the normal allele (Bernard *et al.*, 1983). It has been postulated that deregulated expression of the translocated allele shuts down transcription of the normal allele (Leder *et al.*, 1983). Evidence that the domains required for autosuppression are identical to those required for transformation supports this theory (Penn *et al.*, 1990b).

1.4.3 The c-Myc Protein.

The c-*myc* gene encodes two major protein products of 439 and 453 amino acids, with molecular weights of 64 and 67 kilodaltons (Hann and Eisenman, 1984). The two protein products are derived from independent translational initiation sites in the same reading frame, the p64 protein initiated at the first AUG in exon 2 and p67 initiated at the 3' end of exon 1 (Hann *et al.*, 1988). The p64 polypeptide is the major translation product found in normal cells (Hann and Eisenman, 1984). Rearrangements in exon 1, which occur in many tumour types, result in the loss of the p67 initiation site and can alter the ratio of p64 and p67 proteins in these tumour cells (Hann *et al.*, 1988).

The c-Myc polypeptides are nuclear phosphoproteins with short half-lives of 20-30 minutes which localise to the nucleus and can bind to sequence specific DNA (Hann and Eisenman, 1984; Stone *et al.*, 1987; Dang and Lee, 1988; Watt *et al.*, 1985; Blackwell *et al.*, 1990). A number of evolutionary conserved regions are important for the function of the c-Myc protein (Stone *et al.*, 1987; Penn *et al.*, 1990b; Kato and Dang, 1992). The carboxy-terminus which is the most highly conserved domain (Stone *et al.*, 1987), has been shown to contain nuclear localisation signals (Dang and Lee, 1988), a basic region important for sequence specific DNA binding, and a helix-loop-helix leucine zipper motif (bHLH-LZ) essential for protein dimerisation (Landschulz *et al.*, 1988; Murre *et al.*, 1989). The c-Myc protein also contains a functional transactivation domain (Lech *et al.*, 1988) which has three independent activation regions and has been mapped to the amino-terminus (Kato *et al.*, 1990). These regions, required for the biological activity of c-Myc

(Stone *et al.*, 1987), are characteristic of transcription factors which led to the suggestion that c-Myc was involved in transcriptional regulation (Collum and Alt, 1990; Luscher and Eisenman, 1990). Sequence specific transactivation by c-Myc has been demonstrated confirming the role of c-Myc as a transcription factor (Amin *et al.*, 1993; Kretzner *et al.*, 1992; Gu *et al.*, 1993). A role for c-Myc in promoting DNA replication has also been suggested in which c-Myc binds to the initiation site of replication (Iguchi-Ariga *et al.*, 1987).

1.4.4 Max and the Mad Family Proteins.

The c-Myc proteins (as well as N-Myc and L-Myc) form stable heterodimers with Max, another bHLH-LZ protein which shares homology to c-Myc (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991). Max is a highly conserved stable nuclear protein which is constitutively expressed throughout the cell cycle (Wagner *et al.*, 1992). Myc and Max dimerise through the bHLH-LZ domains and bind to the DNA sequence CACGTG through the bHLH-LZ domain of c-Myc (Blackwell *et al.*, 1990; Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991). Dimerisation of c-Myc to Max is essential for all known c-Myc functions including c-Myc transcriptional activation, c-Myc induced transformation and c-Myc mediated apoptosis (Amati *et al.*, 1992; Prendergast *et al.*, 1991; Amati *et al.*, 1993). Overexpression of Max can suppress c-Myc/Max transactivation (Amati *et al.*, 1992) as Max, which lacks a transactivation domain, can form homodimers which compete with c-Myc/Max dimers to form DNA binding complexes (Gu *et al.*, 1993; Amin *et al.*, 1993).

A number of other Myc-like proteins have recently been identified which also form dimers with Max (reviewed by Amati and Land, 1994; Henriksson and Luscher, 1996). The best characterised of the Mad family proteins are the Mad-1 and Mxi-1 proteins. Mad-1 and Mxi-1 are bHLH-LZ proteins which bind the same DNA consensus sequence when bound to Max as c-Myc/Max dimers (Ayer *et al.*, 1993; Zervos *et al.*, 1993). Mad family proteins, which are expressed at low levels in proliferating cells and high levels during differentiation (Larsson *et al.*, 1994; Chin *et al.*, 1995), may antagonize c-Myc function by competing with c-Myc for the interaction of Max during inhibition of growth

and induction of differentiation (Ayer *et al.*, 1993; Zervos *et al.*, 1993; Henriksson and Luscher, 1996). Evidence suggests that Mad-1 and Mxi-1 may mediate antagonist activities in part through a putative transcriptional repressor (reviewed by Henriksson and Luscher, 1996). Expression of Mad has been found to repress the activity of c-Myc and can suppress oncogenic transformation (Ayer *et al.*, 1993; Chin *et al.*, 1995; Chen *et al.*, 1995).

1.4.5 c-Myc, Transcriptional Regulation and Cell Cycle Control.

Expression of the c-*myc* oncogene is invariably associated with cellular proliferation and is downregulated during growth arrest and differentiation (as discussed above and reviewed by Marcu *et al.*, 1992; Henriksson and Luscher, 1996). An immediate early response gene, c-*myc* is essential for progression through the G1 phase of the cell cycle. Induced expression of c-*myc* can also override G1 growth arrest mediated by the *RB* and p53 tumour suppressor genes (Goodrich and Lee, 1992; Hermeking *et al.*, 1995). It has been proposed that the action of c-Myc on the cell cycle is through the transcriptional regulation of genes involved in cellular proliferation (Collum and Alt, 1990; Luscher and Eisenman, 1990). It is unclear how c-Myc interacts with the basal transcriptional machinery, however the recent demonstration that c-Myc binds to the TATA-binding protein (TBP) of the TFIID transcription initiation complex may provide one possible mechanism (Maheswaran *et al.*, 1994). The transcriptional activation of c-Myc may be regulated, at least in part, by the pRb-related p107 protein which can bind to and suppress the transcriptional activation domain of the c-Myc protein (Gu *et al.*, 1994).

Expression of c-Myc has been correlated with the induction and repression of a number genes including the modulation of its own gene expression (Penn *et al.*, 1990a). Target genes which are responsive to c-Myc include the *α-prothymosin* gene, the *ornithine decarboxylase* (ODC) gene, the p53 tumour suppressor gene, the *ECA39* gene and the *cad* gene (reviewed by Henriksson and Luscher, 1996). These genes all possess c-Myc/Max response elements. It is anticipated that the number of genes which are regulated by c-Myc may be very large. Recent reports have indicated that c-*myc* may mediate its action on the cell cycle by affecting the expression of G1-phase cyclins, cyclin

dependent kinases and the cell cycle phosphatase cdc25, therefore allowing progression through the restriction point of the cell cycle (Daksis *et al.*, 1994; Hanson *et al.*, 1994; Steiner *et al.*, 1995; Galaktionov *et al.*, 1996). Activation of cyclin D1-cdk4 and cyclin E-cdk2 complexes by c-Myc result in the phosphorylation of the pRb protein and cell cycle progression (Hanson *et al.*, 1994; Steiner *et al.*, 1995).

1.4.6 c-Myc and Apoptosis.

In addition to its role in mediating cellular proliferation, the c-Myc protein induces apoptosis in a number of cell types under conditions of restrained cell growth (Askew *et al.*, 1991; Evan *et al.*, 1992) and is required for T-cell receptor stimulated apoptosis in T-cell hybridomas (Shi *et al.*, 1992). Apoptosis is a physiological function of c-Myc which occurs at various points in the cell cycle (Evan *et al.*, 1992) and can be inhibited by specific cytokines (Harrington *et al.*, 1994). Apoptosis induced by c-Myc is also abrogated by expression of the *bcl*-2 survival gene (Fanidi *et al.*, 1992; Bissonnette *et al.*, 1992). The domains of the c-Myc protein required for the execution of the apoptotic pathway are identical to those domains necessary for transformation, transactivation and DNA binding, specifically the bHLH-LZ and transactivation domains (Evan *et al.*, 1992; Stone *et al.*, 1987). Dimerisation with Max is also essential for c-Myc induced apoptosis (Amati *et al.*, 1993; Bissonnette *et al.*, 1994). The ODC gene which is a direct target of c-Myc transactivation has been shown to be a mediator of c-Myc-induced apoptosis (Packham and Cleveland, 1994). Several reports have also demonstrated that the p53 tumour suppressor gene may be required for c-Myc induced apoptosis, at least in certain cell types (Hermeking and Eick, 1994; Wagner *et al.*, 1994; Saito and Ogawa, 1995). The apoptotic function of the c-Myc protein cannot be separated from its role in cellular proliferation. It has been suggested that the outcome of these conflicting signals in the cell context is modulated by other cellular events (Evan and Littlewood, 1993). Proteins which inhibit apoptosis therefore would be expected to be potent *myc* collaborators in tumourigenesis, as has been found with the *bcl*-2 oncogene (Fanidi *et al.*, 1992; Bissonnette *et al.*, 1992).

1.4.7 c-*myc* and Tumourigenesis.

Deregulated expression of the c-*myc* oncogene, which occurs by a variety of mechanisms (reviewed by Cole, 1986; Eisenman, 1989), results in the promotion of unrestrained growth. The domains required for the transforming properties of the c-*myc* oncogene are the same as those important for the functional activity of c-*myc* (Stone *et al.*, 1987). Abnormal c-*myc* expression can result from chromosomal translocations, gene amplification and proviral insertions in a variety of tumour types. Amplification of the c-*myc* gene has been observed in a number of human tumours and is particularly associated with advanced stages of malignancy (Little *et al.*, 1983; Yokota *et al.*, 1986). Amplification of the N-*myc* oncogene has similarly been associated with aggressive neuroblastomas (Brodeur *et al.*, 1984). In Burkitt's lymphoma and mouse plasmacytomas, specific chromosomal translocations link the c-*myc* gene to the regulatory elements of immunoglobulin genes (Taub *et al.*, 1982; Leder *et al.*, 1983). Exon 1 is often deleted or mutated during these translocations giving rise to deregulated c-*myc* transcriptional regulation and elevated expression (Spencer and Groudine, 1991). The loss of exon 1 may effect the stability of c-*myc* mRNA which could account for the increased levels of c-*myc* in Burkitt's lymphoma (Rabbitts *et al.*, 1985). Expression levels in the transformed cells were found to be as high, but not necessarily higher than the levels of mRNA in normal proliferating cells (Keath *et al.*, 1984). Therefore, deregulation of the c-*myc* gene in these tumours resulted in constitutive expression rather than overexpression.

Proviral insertion by slow transforming retroviruses in murine and feline T-cell lymphomas and in avian B-cell lymphomas frequently results in deregulated c-*myc* expression (Selten *et al.*, 1984; Neil *et al.*, 1984; van Lohuizen and Berns, 1990). Insertion of retroviral sequences near to, or within the c-*myc* locus leads to disruption of exon 1 and the normal transcriptional regulation of c-*myc*. This was first observed in avian leukosis virus (ALV)-induced lymphomas when the ALV provirus inserted its promoter sequences adjacent to the endogenous c-*myc* gene, placing the cellular gene under the control of the viral promoter and augmenting c-*myc* expression (Hayward *et al.*, 1981).

The transforming ability of c-*myc* has been confirmed using cell culture studies and transgenic animals. Transfection of v-*myc* and rearranged c-*myc* genes can immortalise rat embryo fibroblast cells (Mougneau *et al.*, 1984). In addition the c-*myc* gene has been shown to cooperate with an activated *ras* oncogene to transform primary cells (Land *et al.*, 1983). Constitutive expression of c-*myc* in transgenic mice has provided direct evidence that this proto-oncogene can contribute to tumourigenesis in a number of cell types (Adams *et al.*, 1985; Stewart *et al.*, 1984a; Adams and Cory, 1991a; Morgenbesser and DePinho, 1994). These transgenic models will be discussed in section 1.7.

1.5 TUMOUR SUPPRESSOR GENES.

Tumour suppressor genes, also referred to as anti-oncogenes and recessive oncogenes, are involved in the negative regulation of cell growth. The normal function of these genes can be thought of as the antithesis to the role of oncogenes which act to positively regulate cellular proliferation. Loss of wild type function of these genes provides a distinct growth advantage during cellular transformation. Tumour suppressor genes have been found to be functionally inactivated in a variety of human tumours. Mutations at tumour suppressor loci are however generally recessive to the normal allele and require functional loss of both copies of the gene in order to promote tumourigenesis. Indeed it has been the characteristic loss of heterozygosity at tumour suppressor loci, involving deletion of one allele and mutation of the remaining allele, that has become the classical way of identifying these genes in human cancers (Ponder, 1988). A number of tumour suppressor genes have now been identified and characterised. The products of these genes function in a variety of different ways from transcriptional activation to cell adhesion in order to control normal cell growth (Skuse and Ludlow, 1995).

The concept of tumour suppressor genes originated from studies by Knudson on the statistical analysis of the childhood cancer retinoblastoma. Knudson proposed that the development of both hereditary and sporadic forms of retinoblastoma required two mutational events (Knudson, 1971), and that the same genetic mutations were required

for both forms (Knudson, 1985). It was suggested that the early onset of bilateral, multifocal tumours characteristic of hereditary retinoblastoma was the result of an inherited germline mutation and second mutation occurring in a limited number of somatic cells. In non-hereditary retinoblastoma, two somatic mutations would have to occur in a single cell consistent with the later onset of unilateral sporadic tumours. Comings extended Knudson's hypothesis by suggesting that the two mutations should occur in both alleles of the same regulatory gene, with the first mutation recessive to the normal allele (Comings, 1973). Historically, two lines of investigation have led to the identification of tumour suppressor genes, somatic cell hybridisation studies and genetic analysis of familial and sporadic human cancers.

1.5.1 Cell Hybrids.

A key observation made from experiments on somatic cell hybridisation was that fusion of a malignant cell with a normal cell often produced a hybrid in which the malignant phenotype was suppressed (Harris, 1988; Stanbridge and Cavenee, 1989). It was deduced that repression of the tumourigenic state was due to genetic information inherent to the normal cells. Reversion to the tumourigenic state could be induced by subsequent chromosomal loss from the hybrid cell, indicative of suppressor activity at the lost site (Harris, 1988). Microcell fusion experiments involving the transfer of single chromosomes from normal cells to cancer cells have provided a direct test to assess the suppressive nature of specific chromosomes. Introduction of human chromosome 11 for example, can suppress the tumourigenic phenotype of a tumour cell line (Weissman *et al.*, 1987).

1.5.2 Tumour Suppressor Genes and Human Cancers.

Cytogenetic and DNA polymorphic analyses of familial cancers have provided much of the evidence to support the existence of tumour suppresssor genes. Germline loss of genetic material at specific chromosomal loci has been demonstrated for various hereditary cancers including retinoblastoma, Wilms' tumour, neurofibromatosis and colorectal carcinoma (reviewed by Stanbridge and Cavenee, 1989; Marshall, 1991; Skuse

and Ludlow, 1995). Furthermore, loss of heterozygosity involving somatic mutation or deletion of the normal allele often occurs at these loci in the tumours of affected individuals (Ponder, 1988). It is the absence of functional genes identified at these tumour suppressor loci which contributes to the tumourigenic phenotype of familial cancers. A variety of non-familial malignancies have also demonstrated homologous allelic inactivation at specific chromosomal sites (Ponder, 1988). In tumours where both hereditary and sporadic forms exist it is generally the same chromosomal locus which is inactivated, although in sporadic cancer both alleles are inactivated somatically. The first tumour suppressor gene to be identified was the retinoblastoma susceptibility gene (*RB*). Consistent with Knudson's hypothesis, familial retinoblastoma had a germline mutation which predisposed to neoplasia, with homologous loss of alleles at this locus in retinoblastoma tumours (Cavenee *et al.*, 1983). A functional gene was mapped to this locus which was inactivated in the tumours, but not normal tissues, of affected individuals (Friend *et al.*, 1986; Lee *et al.*, 1987a).

Similar to retinoblastoma, homologous loss of alleles was demonstrated in patients with sporadic and familial Wilms' tumour, an embryonal malignancy of the kidney (Koufos *et al.*, 1984; Fearon *et al.*, 1984). However more than one tumour suppressor locus may be involved in Wilms' tumour making it more complex than retinoblastoma. Two loci on chromosome 11, 11p13 and 11p15, and a site on chromosome 16 have been implicated in Wilms' tumourigenesis (Henry *et al.*, 1989; Maheswaran and Haber, 1995). Although multiple genetic loci have been associated with Wilms' tumour, only one tumour suppressor gene has been extensively characterised. The Wilms' tumour gene (*WT1*), localised to chromosome 11p13, is found to be deleted in Wilms' tumours (Rose *et al.*, 1990; Gessler *et al.*, 1990; Call *et al.*, 1990) and when re-introduced into a Wilms' tumour cell line can suppress the tumourigenic phenotype (Weissman *et al.*, 1987). The *WT1* gene encodes a zinc-finger protein (Call *et al.*, 1990; Gessler *et al.*, 1990) which functions as a repressor of gene transcription, a property which is lost in tumour cells (Madden *et al.*, 1991; Drummond *et al.*, 1992).

Allelic deletions at chromosome 17 led to the hypothesis that a tumour suppressor gene was involved in the development of neurofibromatosis type 1 (NF1), the most common

hereditary condition predisposing to neoplasia (Skuse *et al.*, 1989). The *NF1* gene, localised to chromosome 17p11 (Barker *et al.*, 1987; Fountain *et al.*, 1989; O'Connell *et al.*, 1989), was found to encode a GTPase-activating protein, neurofibromin, which interacted with the *ras* gene product (Xu *et al.*, 1990). Neurofibromin acts by converting the active form of RAS to the inactive form, consequently inhibiting RAS stimulation of cell proliferation (Xu *et al.*, 1990). More than fifty germline *NF1* mutations have been described which are presumed to disrupt or inactivate neurofibromin (Colman and Wallace, 1995). Specific loss of alleles from chromosome 22 (Seizinger *et al.*, 1987) suggested that inactivation of a tumour suppressor gene was also involved in neurofibromatosis type 2 (NF2), a distinct autosomal disorder from NF1 (Colman and Wallace, 1995). The *NF2* tumour suppressor gene located at chromosome band 22q11 (Seizinger *et al.*, 1987; Trofatter *et al.*, 1993), encodes a protein, merlin, which shares homology with a family of proteins proposed to link cytoskeletal components with proteins in the cell membrane (Trofatter *et al.*, 1993). Merlin is expressed widely in normal cells and deleted in NF2 tumours.

Several tumour suppressor loci have been identified in hereditary and sporadic colorectal carcinoma. Loss of a region of chromosome 5q in colorectal tumours of patients with familial adenomatous polyposis (FAP), an inherited disorder characterised by the presence of multiple colonic polyps, suggested involvement of a putative tumour suppressor gene at this locus (Leppert *et al.*, 1987; Bodmer *et al.*, 1987). The FAP gene, *APC* (adenomatous polyposis coli) was cloned and shown to be inactivated in the early stages of both FAP and sporadic colorectal cancer (Nishisho *et al.*, 1991; Kinzler *et al.*, 1991). The *APC* gene product associates with β-catenin and may facilitate the interaction between catenins and cadherin, a cell surface protein important for cellular adhesion (Su *et al.*, 1993). Enhanced intercellular adhesion may prevent independent growth of epithelial cells. It has been proposed that APC may play a role in intercellular communication via enhanced cellular adhesion and also in intracellular communication from the cell surface to the site of microtubule formation (Dunlop, 1995). Recognition of homologous allelic loss at two other chromosomes, 17 and 18, suggested that more than one tumour suppressor gene was involved in FAP (Law *et al.*, 1988; Fearon and Vogelstein, 1990). Allelic deletions of these loci were found to be a frequent event in

colorectal carcinoma and occurred at a late stage in the progression of tumourigenesis (Fearon and Vogelstein, 1990; Fearon *et al.*, 1990). The p53 tumour suppressor gene was identified as the gene at position 17p (Baker *et al.*, 1989), and *DCC* (deleted in colorectal cancer) as the gene at chromosome 18q (Fearon *et al.*, 1990). Indicative of a tumour suppressor gene, *DCC* was expressed in most normal tissues including colonic mucosa, but was greatly reduced or absent in most colorectal carcinomas. The *DCC* gene product is found to be a cell surface protein and has been implicated in the promotion and maintenance of normal cellular differentiation (Hedrick *et al.*, 1994). Loss of alleles at chromosomes 5, 17 and 18 have been described for sporadic colorectal carcinoma as well as for FAP (Solomon *et al.*, 1987; Vogelstein *et al.*, 1989; Fearon and Vogelstein, 1990).

A second, more common, form of familial colorectal carcinoma is hereditary nonpolyposis colorectal cancer (HNPCC). Two tumour suppressor loci have been identified for HNPCC, *MSH2* at chromosome 2 (Peltomaki *et al.*, 1993; Fishel *et al.*, 1993) and *MLH1* at chromosome 3p (Papadopoulos *et al.*, 1994). Both of these genes have been implicated in DNA repair mechanisms suggesting that mutations in these genes may lead to an increased accumulation rate of spontaneous mutations (Dunlop, 1995).

Genetic linkage studies have led to the identification of two breast cancer genes which confer susceptibility to hereditary breast cancer, *BRCA1* on chromosome 17q (Miki *et al.*, 1994) and *BRCA2* on chromosome 13q (Wooster *et al.*, 1994). Mutation of the *BRCA1* tumour suppressor gene is thought to affect half of those families with an inherited breast cancer syndrome and most families with early-onset breast and ovarian cancer syndrome (Miki *et al.*, 1994). *BRCA2* has been associated with early-onset hereditary breast cancer and male breast cancer but has a lower ovarian cancer risk than *BRCA1* (Wooster *et al.*, 1994). Loss of heterozygosity at the *BRCA1* and *BRCA2* loci have been described for both familial and sporadic breast cancers (Bishop, 1995; Cleton-Jansen *et al.*, 1995; Collins *et al.*, 1995).

Some tumour suppressor loci have been associated with more than one form of cancer. Allelic deletions of chromosome 3 for example, were reported in renal cell carcinoma

(Zbar *et al.*, 1987; Kovacs *et al.*, 1988) and small cell lung cancer (Naylor *et al.*, 1987). The p53 tumour suppressor gene has also been associated with an extensive number of sporadic and hereditary cancers (Nigro *et al.*, 1989; Hollstein *et al.*, 1991; Greenblatt *et al.*, 1994). Loss of p53 has been implicated in the familial Li-Fraumeni syndrome (Malkin *et al.*, 1990; Srivastava *et al.*, 1990) and may be important for the development of malignant, but not benign, hereditary NF1 tumours (Menon *et al.*, 1990) and for the progression of familial colorectal carcinoma (Fearon and Vogelstein, 1990). The role of p53 in Li-Fraumeni syndrome and sporadic tumours is discussed in section 1.6.2.

More recent has been the identification of the *MTS1* (multiple tumour suppressor 1) gene, localised to chromosome 9p21. *MTS1* was found to be inactivated in a variety of tumour cell lines by homozygous deletion or mutation (Kamb *et al.*, 1994) or by DNA methylation (Merlo *et al.*, 1995). The *MTS1* gene encodes a cell cycle protein, p16, which has been shown to negatively regulate cell cycle progression by binding to and inhibiting cyclin-dependent kinase 4 (cdk4) activity (Serrano *et al.*, 1993).

1.5.3 The Retinoblastoma Tumour Suppressor Gene.

The retinoblastoma susceptibility gene (*RB*) is probably the best characterised of all the tumour suppressor genes. The *RB* gene has been found to be functionally inactivated in both hereditary and sporadic forms of retinoblastoma (reviewed by Gallie *et al.*, 1995; Goodrich and Lee, 1990), and in a limited number of sporadic tumour types including breast cancer, small cell lung cancer, osteosarcomas and soft tissue sarcomas (Lee *et al.*, 1988; Harbour *et al.*, 1988; Horowitz *et al.*, 1990; Weichselbaum *et al.*, 1988). Consistent with loss of heterozygosity at chromosomal position 13q14 in retinoblastomas (Cavenee *et al.*, 1983), the *RB* gene was mapped to this locus (Friend *et al.*, 1986; Lee *et al.*, 1987a). *RB* was found to be expressed in a variety of tissues, including normal retina, but was absent in retinoblastoma tumours and cell lines (Friend *et al.*, 1986; Lee *et al.*, 1987a; Horowitz *et al.*, 1990). These studies suggested that inactivation of *RB* was important for deregulated cell growth and tumourigenesis. This was confirmed by Huang *et al.*, (1988) who demonstrated that replacement of wild type *RB* into a retinoblastoma cell line lacking functional *RB*, resulted in growth suppression and a

reduction in tumourigenicity. Furthermore, the transforming abilities of several DNA tumour viruses were found to be dependent on the inactivation of *RB* through the binding of viral oncogenes to the *RB* gene product (Whyte *et al.*, 1988; DeCaprio *et al.*, 1988; Dyson *et al.*, 1989). These studies suggested that oncogenic viruses may cause cancers by sequestering the products of tumour suppressor genes.

The *RB* gene product (pRb), a nuclear phosphoprotein with DNA binding affinity (Lee *et al.*, 1987b), negatively regulates cell proliferation by inhibiting cell cycle progression (Goodrich *et al.*, 1991). The pRb protein exists in multiple phosphorylation states; underphosphorylated in the G0/G1 phase of the cell cycle and hyperphosphorylated in S, G2 and M phases (DeCaprio *et al.*, 1989; Mihara *et al.*, 1989; Ludlow *et al.*, 1990). The function of pRb is regulated by cell cycle-dependent phosphorylation with protein phosphorylation coincident with the onset of DNA replication (DeCaprio *et al.*, 1989; Mihara *et al.*, 1989; Ludlow *et al.*, 1990). Modulation of pRb phosphorylation occurs via cyclin-dependent kinases, cdks (Hinds *et al.*, 1992; Akiyama *et al.*, 1992; Gallie *et al.*, 1995). Significantly, the p16 tumour suppressor protein was found to inhibit pRb phosphorylation by blocking cdk4 activity (Serrano *et al.*, 1993), suggesting that p16 and pRb may act on the same tumour suppressor pathway. This hypothesis would explain why inactivation of both p16 and pRb proteins do not tend to occur in the same tumour cells but rather exhibit an inverse relationship (Whitaker *et al.*, 1995; Kaye *et al.*, 1995).

It has been proposed that pRb functions to control gene transcription in association with the cellular E2F transcription factor (Chellappan *et al.*, 1991; Weintraub *et al.*, 1992; Nevins, 1992). Unphosphorylated pRb, when bound to E2F in G1, interferes with the transcriptional activation capacity of E2F, preventing transcription of genes that are required for cell proliferation. The pRb protein has been shown to have a role in the transcriptional regulation of certain cellular growth control genes including c-*fos*, interleukin 6, cyclin D1 and c-*myc* (Robbins *et al.*, 1990; Santhanam *et al.*, 1991; Muller *et al.*, 1994; Adnane and Robbins, 1995). Physical interaction of viral oncoproteins with pRb (Whyte *et al.*, 1988; DeCaprio *et al.*, 1988; Dyson *et al.*, 1989) functionally inactivates pRb by blocking association of pRb with E2F thereby promoting transcriptional activation of genes important for cell cycle progression (Nevins, 1992).

Binding of the cellular oncoprotein MDM2 to the pRb protein can also perturb the growth regulatory function of pRb and relieve suppression of E2F transcriptional activity (Xiao *et al.*, 1995). The transcriptional regulation of gene expression by pRb in G1 of the cell cycle is an important aspect of pRb tumour suppression. Recent investigations however have indicated that the pRb protein may also play a role in suppressing apoptosis (Haas-Krogan *et al.*, 1995; Haupt *et al.*, 1995). Consistent with these observations, extensive apoptosis was reported to occur in mice which lacked a functional *RB* gene (Clarke *et al.*, 1992). The pRb protein can therefore function to inhibit proliferation, tumourigenesis and apoptosis.

1.6 THE P53 TUMOUR SUPPRESSOR GENE.

1.6.1 Background.

The p53 phosphoprotein, initially classified as a tumour specific antigen, was first identified as a cellular protein which was bound by SV40 large T antigen in virally transformed murine cells (Lane and Crawford, 1979; Linzer and Levine, 1979) and was present in chemically induced sarcomas and spontaneously transformed fibroblasts (DeLeo *et al.*, 1979). An accumulation of the p53 protein was subsequently described in a range of spontaneous, chemically and virally induced murine and human tumours, and transformed cell lines (Crawford *et al.*, 1981; Rotter, 1983; Crawford, 1983). Although early reports had been unable to detect p53 in normal tissues, it was found to be present albeit at much lower levels than in transformed cells (Dippold *et al.*, 1981; Benchimol *et al.*, 1982; Crawford, 1983). This is due to a very short half life of the protein in normal cells and post-translational stabilisation in transformed cells (Oren *et al.*, 1981; Crawford, 1983; Rogel *et al.*, 1985). The p53 protein was thought to be a dominant oncogene with reports that genomic and cDNA clones of p53 could immortalise cells of finite lifespan (Jenkins *et al.*, 1984; Rovinski and Benchimol, 1988) and cause immortalised cells to become tumourigenic (Eliyahu *et al.*, 1985). Furthermore, these p53 clones were shown to co-operate with the *ras* oncogene in the transformation of primary rat fibroblasts (Jenkins *et al.*, 1984; Parada *et al.*, 1984). However, it transpired

that the p53 clones which co-operated with *ras* to transform cells were mutant forms of the wild type protein and that wild type p53 did not have a transforming role in co-operation with *ras* (Finlay *et al.*, 1988; Hinds *et al.*, 1989). Indeed wild type p53 was found to inhibit oncogene-mediated cellular transformation (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989).

Reports that the p53 protein could suppress transformation and that point mutations in the gene abrogated this activity, led to the suggestion that the wild type p53 gene may function as a recessive oncogene or tumour suppressor gene (Finlay *et al.*, 1989; Eliyahu *et al.*, 1989; Lane and Benchimol, 1990). Several lines of investigation were consistent with this hypothesis. It had been observed that the p53 gene was frequently mutated and inactivated in Friend virus-induced murine erythroleukaemia cell lines suggesting that loss of p53 activity, rather than gain of activity, was important for the malignant transformation of these cells (Mowat *et al.*, 1985; Chow *et al.*, 1987; Ben-David *et al.*, 1988). The p53 gene was similarly inactivated in cell lines transformed by Abelson murine leukaemia virus (Wolf and Rotter, 1984). Moreover, Vogelstein and colleagues reported that both alleles of the p53 gene were inactivated in colorectal cancer, a trademark of tumour suppressor genes (Baker *et al.*, 1989). This group further determined that the p53 gene was frequently deleted and mutated in a range of diverse tumour types (Nigro *et al.*, 1989). Transfection of wild type protein into a variety of cell types inhibited cellular proliferation confirming that p53 could act to suppress cell growth (Baker *et al.*, 1990b; Chen *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990). Definitive proof of tumour suppressor activity was later provided by Donehower and colleagues with the generation of mice deficient for endogenous p53 function (Donehower *et al.*, 1992). These animals developed normally suggesting that the p53 gene was not essential for development but were highly susceptible to spontaneous tumour development in a range of different tissues.

1.6.2 p53 and Human Cancer.

Alterations at the p53 tumour suppressor gene have been described for an extensive number of tumour types (Nigro *et al.*, 1989; Bartek *et al.*, 1991; Hollstein *et al.*, 1991;

Jego *et al.*, 1993; Greenblatt *et al.*, 1994). The number of reports detailing mutations of p53 in human tumours has been overwhelming and has earned p53 the reputation as the most commonly altered genetic lesion in human cancer. By contrast, most other tumour suppressor genes are inactivated in only one or a few tumour types. This suggests that p53 plays a critical role in controlling oncogenesis in numerous tissues. Point mutations, deletions and aberrant expression of the p53 gene have been described for colorectal carcinoma, breast carcinoma, lung cancer, head and neck tumours, leukaemia and others (Baker *et al.*, 1989; Bartek *et al.*, 1990; Cheng and Haas, 1990; Iggo *et al.*, 1990; Ahomadegbe *et al.*, 1995). Inactivation of p53 has also been linked to the hereditary Li-Fraumeni syndrome (LFS), a familial condition which predisposes kindred at an early age to a range of tumour types including breast cancer, sarcomas, brain tumours and leukaemia. Kindred often develop two or more of these tumour types. Two groups of workers independently reported that LFS was associated with an inherited mutation in the p53 tumour suppressor gene (Malkin *et al.*, 1990; Srivastava *et al.*, 1990). Furthermore, tumours of affected individuals had lost the remaining wild type p53 allele. The spectrum of tumours in LFS is consistent with the spectrum of sporadic tumours associated with loss of p53 function (Malkin *et al.*, 1990; Srivastava *et al.*, 1990).

As is characteristic of tumour suppressor genes, loss of p53 function is often associated with inactivation of both alleles. This has been reported for several tumour types where loss of one p53 allele is accompanied by mutation or deletion at the second allele (Nigro *et al.*, 1989; Baker *et al.*, 1989; Sakashita *et al.*, 1992). Loss of heterozygosity at the p53 locus has been found to be important for neoplastic progression, often occurring as a late event in tumourigenesis and correlated with poor clinical prognosis (Baker *et al.*, 1990a; Malkin *et al.*, 1990; Ichikawa *et al.*, 1992; Dohner *et al.*, 1995). Alterations at the p53 gene can also occur as an early event, as has been described for small cell lung cancers and head and neck tumours (Kaye *et al.*, 1995; Ahomadegbe *et al.*, 1995).

1.6.3 Interaction of p53 with Viral and Cellular Proteins.

The p53 gene, like the *RB* gene, was found to be inactivated in virally transformed cells. SV40 large T antigen, adenovirus E1B 55kDa protein and human papillomavirus (HPV)

types 16 and 18 E6 proteins all complex with the p53 protein (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow *et al.*, 1982; Werness *et al.*, 1990). SV40 large T antigen and E1B 55kDa protein stabilise the p53 protein and sequester its tumour suppressor activity (Lane and Crawford, 1979; Levine, 1990; Yew and Berk, 1992) while the HPV E6 protein promotes p53 protein degradation (Scheffner *et al.*, 1990). There is no selection for p53 gene mutations in virally transformed cells as the viral proteins neutralize the tumour suppressor activity of wild type p53. This is seen with cervical carcinoma cell lines where the presence of mutant p53 proteins is detected in HPV negative lines but not in HPV positive cell lines (Scheffner *et al.*, 1991).

Similar to the action of viral oncoproteins, a cellular protein has been identified which complexes with wild type p53 and inactivates its tumour suppressor function (Barak and Oren, 1992; Oliner *et al.*, 1992; Momand *et al.*, 1992). The *murine double minute* 2 (*MDM2*) oncogene has been found to be amplified in several human sarcomas (Oliner *et al.*, 1992). It was reported that overexpression of MDM2 protein exists only in cells with wild type p53 and does not co-exist in cells with mutant p53 (Oliner *et al.*, 1992; Zhou *et al.*, 1995). MDM2 may provide an alternative method of inactivating the p53 tumour suppressor gene in spontaneously occurring tumours.

1.6.4 p53 Gene Structure and Protein Properties.

The highly conserved p53 gene is comprised of 11 exons, the first of which is non-coding, and has been localised to human chromosome 17p13 (Lamb and Crawford, 1986; Isobe *et al.*, 1986; Miller *et al.*, 1986). There are two p53 genes in the mouse genome, a functional gene on chromosome 11 and an inactive pseudogene on chromosome 14 (Czosnek *et al.*, 1984; Rotter *et al.*, 1984). The human p53 gene spans 20kbp and encodes a protein of 393 amino acids with molecular weight 53 kDa as determined by SDS-polyacrylamide gel electrophoresis (Harlow *et al.*, 1985; Lamb and Crawford, 1986). The functional murine gene has 11 exons and encodes a protein of 390 amino acids which, like the human protein, is rich in proline residues (Bienz *et al.*, 1984; Pennica *et al.*, 1984).

The proteins encoded by the mouse, human, *Xenopus laevis* and several other species share a high degree of amino acid sequence homology (Pennica *et al.*, 1984; Harlow *et al.*, 1985; Soussi *et al.*, 1987; Soussi, 1995). The p53 protein consists of a highly charged acidic amino terminus, a hydrophobic proline rich central region and a highly charged basic carboxy-terminus. A cluster of three nuclear localisation domains (Shaulsky *et al.*, 1990b; Shaulsky *et al.*, 1991b) and an oligomerisation domain (Milner *et al.*, 1991; Shaulian *et al.*, 1992) have been located at the carboxy-terminus of the protein which also exhibits DNA binding activity (Steinmeyer and Deppert, 1988; Kern *et al.*, 1991; Shohat Foord *et al.*, 1991; Foord *et al.*, 1993). A transactivation domain has been mapped to the p53 amino terminus (O'Rourke *et al.*, 1990; Fields and Jang, 1990; Raycroft *et al.*, 1990). Five regions of the p53 protein have been highly conserved throughout evolution (Soussi, 1995). These regions, referred to as domains I, II, III, IV and V, are frequent targets for 'hot spot' mutations in human malignancies (Nigro *et al.*, 1989; Hollstein *et al.*, 1991). Indeed, 95% of mutations recorded in human tumours are located in the central region of the protein which contains four of the five highly conserved domains (Soussi, 1995). A sequence specific DNA binding domain has been mapped to the highly conserved central core of p53 (Pavletich *et al.*, 1993; Bargonetti *et al.*, 1993; Wang *et al.*, 1993c). The DNA binding function of p53 is necessary for tumour suppressor activity and can be activated by phosphorylation, carboxy-terminus truncation and small peptides (Hupp *et al.*, 1992; 1995).

Although the subcellular location of p53 varies throughout the cell cycle (Shaulsky *et al.*, 1990a), the tumour suppressor activity of p53 requires nuclear localisation (Shaulsky *et al.*, 1991b). Wild type p53, but not mutant p53, functions as a transcription factor activating gene expression when bound to a p53 specific DNA sequence (Kern *et al.*, 1991; Farmer *et al.*, 1992; Kern *et al.*, 1992). Mutant p53 proteins, SV40 large T antigen, adenovirus E1B 55Kb and MDM2 can inhibit p53-mediated transcriptional activation either by blocking DNA binding or by concealing the activation domain (Shaulian et al, 1992; Farmer *et al.*, 1992; Yew and Berk, 1992; Oliner *et al.*, 1993). Genes which do not contain a p53 specific DNA consensus sequence can be transcriptionally repressed by wild type p53 (Ginsberg *et al.*, 1991; Seto *et al.*, 1992). The p53 protein does not directly bind to gene promoters during transcriptional

repression but binds to the TATA binding protein which interferes with the formation of the transcription preinitiation complex (Seto *et al.*, 1992; Ragimov *et al.*, 1993). It has been suggested that the carboxy-terminus of the p53 protein is necessary for transcriptional repression whereas the amino-terminus is important for transcriptional activation (Sang *et al.*, 1994; Shaulian *et al.*, 1995).

Alteration of p53 by mutation generally results in loss of wild type protein function (Lane and Benchimol, 1990; Levine *et al.*, 1991). Several differences between the wild type and mutant proteins exist (Zambetti and Levine, 1993). Wild type p53 functions as a growth suppressor whereas mutant proteins fail to inhibit growth (Hinds *et al.*, 1989; Zambetti and Levine, 1993). Mutant proteins are unable to function as transcriptional activators or transcriptional repressors (Raycroft *et al.*, 1990; Kern *et al.*, 1992; Seto *et al.*, 1992; Deb *et al.*, 1992). In general, wild type p53 has a much shorter half life than the mutant proteins of transformed cells (Crawford, 1983) although the half life of wild type p53 can be prolonged when complexed to viral proteins (Levine, 1990). Mutant p53 proteins have an altered conformational state compared to wild type p53 which can be assessed using monoclonal antibodies (Gannon *et al.*, 1990; Milner and Watson, 1990). Mutant proteins, unlike wild type p53, fail to bind to viral proteins (Levine, 1990) but have been found to form complexes with heat shock proteins (Pinhasi-Kimhi *et al.*, 1986; Sturzbecher *et al.*, 1988). Wild type p53, in contrast does not associate with heat shock proteins (Pinhasi-Kimhi *et al.*, 1986; Sturzbecher *et al.*, 1988). Mutant p53 proteins have been shown to form oligomers with the wild type p53 protein and can sequester its growth suppressing activity in a dominant negative fashion (Gannon *et al.*, 1990; Milner *et al.*, 1991; Shaulian *et al.*, 1992). In this way only one allele would need to be mutated for loss or reduction of suppressor function in human tumours. In addition to loss of function, some p53 mutants have demonstrated a gain of function and can directly promote cellular growth in a dominant oncogenic manner. This was observed when mutant p53 was introduced into cells which lacked endogenous wild type p53 protein resulting in enhanced tumourigenic potential of the cells (Dittmer *et al.*, 1993).

1.6.5 p53, DNA Damage and Cell Cycle Growth Arrest.

Although early studies had suggested a role for p53 in positively modulating growth and cell cycle progression (Milner and Milner, 1981; Mercer *et al.*, 1982; Reich and Levine, 1984; Shohat *et al.*, 1987) it was soon recognised that overexpression of wild type p53 in a variety of cell types inhibited cellular proliferation and abrogated cell cycle progression by inducing a G1 growth arrest (Baker *et al.*, 1990b; Chen *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990; Michalovitz *et al.*, 1990). It has been proposed that the activity of p53 as a promoter and suppressor of growth is regulated by conformational alterations, possibly mediated by phosphorylation (Bischoff *et al.*, 1990; Sturzbecher *et al.*, 1990; Hupp *et al.*, 1992; Picksley *et al.*, 1992; Ullrich *et al.*, 1992). Consistent with its role in suppressing cellular proliferation, p53 has also been found to induce maturation (Kastan *et al.*, 1991b), differentiation (Shaulsky *et al.*, 1991a) and apoptosis (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992).

Over the last few years the role of p53 in the response to DNA damage has been intensively studied. Levels of wild type p53 increase following treatment with UV radiation and other DNA damaging agents (Maltzman and Czyzyk, 1984; Kastan *et al.*, 1991a; Fritsche *et al.*, 1993; Lu and Lane, 1993). It has been proposed that the C-terminus of p53 binds to the damaged DNA which stabilizes the protein accounting for the accumulation of p53 protein levels in damaged cells (Lee *et al.*, 1995). Observations that enhanced levels of p53 were coincident with a reversible G1 growth arrest suggested that p53 functioned as a G1 cell cycle checkpoint following DNA damage (Kastan *et al.*, 1991a; Kuerbitz *et al.*, 1992; Kastan *et al.*, 1992). These studies led Lane (1992) to postulate that p53 acted as a guardian of the genome maintaining DNA integrity in response to genetic insult. By mediating a cell cycle arrest, p53 provided time for DNA to repair prior to cell division. Lane further suggested that cells with inactivated p53 would be unable to elicit the DNA damage response pathway which would result in an accumulation of genetically unstable cells. This hypothesis was supported by studies showing a correlation between loss of p53 cell cycle control and genetic instability (Yin *et al.*, 1992; Livingstone *et al.*, 1992). Re-introduction of wild type p53 into these cells restored cell cycle control and reduced the frequency of gene amplification (Yin *et al.*,

1992). It is probable that p53 operates at more than one point in the cell cycle as recent reports have shown that p53 can induce a G2/M cell cycle block as well as a G1 cell cycle block (Stewart *et al.*, 1995; Aloni-Grinstein *et al.*, 1995; Agarwal *et al.*, 1995).

The p53 response to DNA damage is elicited through the transcription of genes which contain p53-binding sites in their promoters (Lu and Lane, 1993). Several effector genes induced by p53 have been identified. It was demonstrated by Kastan *et al.* (1992) that the human growth arrest and DNA damage-inducible gene, *GADD45*, was induced by p53. *GADD45*, which suppresses cellular growth *in vitro* (Zhan *et al.*, 1994), was found to bind to the proliferating cell nuclear antigen (PCNA) and inhibited entry of cells into S phase (Smith *et al.*, 1994). *GADD45* also stimulated DNA excision repair suggesting a link between p53 and DNA repair (Smith *et al.*, 1994; Smith *et al.*, 1995). Expression of the highly conserved *WAF1* (wild type p53-activated fragment 1) gene was also induced by sequence specific transactivation by p53 (El-Deiry *et al.*, 1993). El-Deiry *et al.* demonstrated that overexpression of the *WAF1* gene could reproduce the growth inhibitory activity of p53. The *WAF1* gene, also referred to as *CIP1*, for cdk-interacting protein, was found to encode a protein, p21, which was a potent inhibitor of G1 cdks (Harper *et al.*, 1993; Xiong *et al.*, 1993). The binding of p21 to cdks prevented phosphorylation of cdk substrates like *RB* and blocked cell cycle progression (Harper *et al.*, 1993; Xiong *et al.*, 1993). Direct evidence that p21 was involved in the p53-mediated DNA damage response was presented in two papers by Dulic *et al.* (1994) and El-Deiry *et al.* (1994). These authors showed a p53 dependent accumulation of p21 which correlated with inhibition of cdk2 kinase activity during radiation-induced G1 growth arrest. Studies using p21 deficient cells corroborated these results by demonstrating that p21 was an essential component of the DNA damage induced p53 G1 growth arrest pathway (Deng *et al.*, 1995; Brugarolas *et al.*, 1995). In addition to its role in cell cycle arrest, p21 has also been shown to inhibit DNA replication through an interaction with the essential DNA replication factor PCNA (Waga *et al.*, 1994; Chen *et al.*, 1995b). The p53 transactivation of p21 and GADD45 may therefore serve two purposes, the first to initiate cell cycle arrest and the other to block replicative DNA synthesis.

Transcriptional activation of other p53 responsive genes may be involved in p53-mediated growth arrest. The *MDM2*, angiogenesis inhibitor thrombospondin-1 and transforming growth factor α genes have been found to be induced by p53 (Wu *et al.*, 1993; Dameron *et al.*, 1994; Shin *et al.*, 1995). Several growth promoting genes have been found to be transcriptionally downregulated by p53 which may also contribute to the cell cycle arrest mediated by p53 in response to DNA damage. Evidence for the downregulation of interleukin-6, c-*fos* and c-*myc* has been described (Santhanam *et al.*, 1991; Ginsberg *et al.*, 1991; Deb *et al.*, 1992; Moberg *et al.*, 1992).

1.6.6 p53 and Apoptosis.

Overexpression of wild type p53 in some tumour cell lines was shown to induce apoptosis (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992; Wang *et al.*, 1993a). Furthermore, p53 was found to be required for DNA damage induced apoptosis. Thymocytes deficient in p53 failed to mediate an apoptotic response induced by radiation and chemotherapeutic DNA-damaging drugs, but were proficient in other forms of apoptosis (Clarke *et al.*, 1993; Lowe *et al.*, 1993a). As in thymocytes, myeloid progenitor cells and intestinal epithelial cells required wild type p53 to elicit an appropriate radiation-induced apoptotic response (Lotem and Sachs, 1993a; Clarke *et al.*, 1994; Merritt *et al.*, 1994). Whole body irradiation of normal animals showed that increased levels of p53 spatially and temporally correlated with apoptosis (Merritt *et al.*, 1994) but that in p53-deficient animals, intestinal cells were completely resistant to radiation with levels of apoptosis not exceeding that of normal background (Clarke *et al.*, 1994; Merritt *et al.*, 1994). The requirement for p53 to execute an efficient apoptosis pathway in response to radiation and anticancer drugs has important implications for cancer therapy as loss of p53 can lead to resistant tumours (Lowe *et al.*, 1993b; 1994b).

The involvement of p53 in apoptotic mechanisms is not restricted to those cells which have undergone genetic damage as reports have documented a role for p53 in the apoptotic response to withdrawal of survival factors (Lotem and Sachs, 1993a) and in oncogene-mediated apoptosis (Debbas and White, 1993; Lowe *et al.*, 1994a; Hermeking

and Eick, 1994; Wagner *et al.*, 1994). The viral oncogene E1A was found to induce apoptosis in a manner dependent on p53 (Debbas and White, 1993; Lowe *et al.*, 1994a). Inactivation of p53 by mutation or binding to E1B was therefore required for efficient viral transformation. The c-*myc* oncogene similarly required wild type p53 to execute its apoptotic pathway in fibroblast cells (Wagner *et al.*, 1994; Hermeking and Eick, 1994). Loss of p53-dependent apoptosis in response to oncogenic events has been shown to be an important determinant of tumour development and tumour progression (Symonds *et al.*, 1994). Symonds and colleagues used a transgenic mouse model to demonstrate that wild type p53 directly suppressed tumour growth by promoting apoptosis of abnormally proliferating cells. Inactivation of p53 in this model resulted in the rapid development of aggressive tumours (Symonds *et al.*, 1994).

The evidence put forward therefore suggests that DNA damage elicits both p53-induced growth arrest and p53-mediated apoptosis. The data available suggests that these are separable functions of p53. Firstly, p53-mediated apoptosis was not dependent on the induction of a growth arrest, although cells in G1 were preferentially susceptible to apoptosis (Yonish-Rouach *et al.*, 1993). Secondly, the p21 protein, an essential component of the p53-induced growth arrest pathway, was not required for p53-induced apoptosis mechanisms (Wagner *et al.*, 1994; Deng *et al.*, 1995; Brugarolas *et al.*, 1995). Indeed, p53-mediated apoptosis has been shown to occur in the absence of p53 activated gene expression (Caelles *et al.*, 1994). However recent investigations indicate that p53 does induce at least some apoptotic pathways through transcriptional activation (Yonish-Rouach *et al.*, 1996). A strong candidate gene for mediating p53-induced apoptosis is the *bax* gene as the direct transactivation of *bax* by p53 has been shown to induce apoptosis in a number of cell types (Miyashita and Reed, 1995). Overexpression of the *bcl*-2 oncogene inhibits p53-mediated apoptosis but not p53-induced growth arrest again suggesting that these are distinct properties of the p53 gene which may be regulated by cellular factors (Chiou *et al.*, 1994). Conversely, overexpression of the c-*myc* oncogene suppressed p53-induced growth arrest but not p53-mediated apoptosis (Saito and Ogawa, 1995; Hermeking *et al.*, 1995). Recent papers have indicated that the fate of p53 may be regulated in part by the *RB* tumour suppressor gene (White, 1994). *RB* has been shown to suppress p53-mediated apoptosis (Haupt *et al.*, 1995) and to direct the

p53-mediated growth arrest response (Slebos *et al.*, 1994). Therefore in the presence of functional *RB*, p53 would promote a G1 growth arrest programme whereas in cells which lacked *RB*, p53 would initiate an apoptotic pathway. These results may explain why certain viruses inactivate both the *RB* and p53 tumour suppressor genes.

1.7 THE USE OF TRANSGENIC MICE IN ONCOLOGY STUDIES.

Since its first application over twelve years ago, transgenic technology has provided some exciting advances in molecular oncology research. Transgenic animals have become very important in directly assessing the role of genes, particularly potential oncogenes, in normal development and tumourigenesis *in vivo*. Cumulative studies have established an unequivocal link between more than thirty genes and neoplastic transformation (Adams and Cory, 1991a; Adams and Cory, 1991b). Transgenic animals further provide a system to test successive events in the progression of disease and to investigate potential therapies. The use of genetically altered animals in modelling human disease is ever increasing and although oncology has been prominent in these studies, it should be noted that good animal models exist for a number of conditions. These include artherosclerosis, diabetes (reviewed in Kappel *et al.*, 1994), pulmonary, immunological and neuropsychiatric disorders (reviewed in Lathe and Mullins, 1993).

1.7.1 Transgenic Technology.

A transgenic animal contains foreign DNA sequences, usually placed there under experimental conditions. Current transgenic technology has progressed to a stage where it is now routinely possible to not only insert exogenous genes, but to interrupt and functionally delete endogenous genes. Furthermore, by directing the transgene sequences to target cells, the effects caused by the introduced genetic alteration can be made tissue or lineage specific. Introduction of foreign gene sequences into mice was first reported by Jaenisch and Mintz (1974) when they microinjected SV40 DNA into the blastocyst cavity of the mouse embryo, albeit without integration into the host DNA. The first experimental germline integration of foreign sequences and subsequent

transmission to progeny was achieved by infecting preimplantation embryos with Moloney murine leukaemia retrovirus (Jaenisch, 1976).

The introduction of exogenous gene sequences into the mouse genome has been achieved by a number of alternative techniques, namely retroviral vectors, pronuclear microinjection and embryonic stem cells. The ability of retroviruses to stably insert their viral genes into the mouse germline, as demonstrated by Jaenisch (1976), provided the basis for the first of these methods. Retroviruses can be used as vectors to insert viral or non-viral genes of interest into the mouse genome. This is achieved by packaging gene sequences into retroviral particles and infecting preimplantation embryos with the recombinant retroviral vector, first demonstrated by van der Putten *et al.* in 1985. Retroviral infection allows the efficient transfer of a single copy of the gene at a single chromosomal site (reviewed in Cameron *et al.*, 1994; Jaenisch, 1988). As infection is usually carried out at a late stage in embryonic development (2 cell to morulae stages), transgenic animals derived using this method are generally mosaic (Jaenisch, 1976; van der Putten *et al.*, 1985). One disadvantage of this method is that there is a limitation to the size of recombinant DNA sequence that can be packaged into the viral vector.

Pronuclear microinjection is the preferred and probably most successful method of generating transgenic animals and involves the direct microinjection of gene sequences into the pronucleus of fertilized mouse embryos (Brinster *et al.*, 1985; Hogan *et al.*, 1986). As pronuclear microinjection is carried out with embryos at a one-cell stage, the inserted gene normally integrates very early during development. As a result, the majority of transgenic animals are not mosaic and are capable of germline transmission. DNA microinjected into either of the two pronuclei is usually found to integrate at a single, presumably random, site. Unlike retroviral vectors, more than one copy of the gene frequently integrates into the host genome. Multiple copies of the DNA molecule, arranged in a tandem head-to-tail array, are generally found at a single chromosomal site (Brinster *et al.*, 1981; Brinster *et al.*, 1985). It has been suggested that recombination of the coinjected DNA molecules occurs before integration (Brinster *et al.*, 1985). Gordon *et al.* (1980) were the first to describe the production of transgenic animals using pronuclear microinjection with the insertion of recombinant DNA sequences of herpes

simplex virus (HSV) and SV40 viral DNA. It was recognized by these authors, and soon after confirmed by others, that DNA introduced by this method was integrated into the mouse germline and passed to progeny in a Mendelian fashion, giving rise to transgenic offspring with the same DNA integration as the parent (Gordon and Ruddle, 1981; Brinster *et al.*, 1981).

Embryonic stem (ES) cells offer a third option for delivering genes into the mouse germline. ES cells are derived from blastocysts and have pluripotent potential (Capecchi, 1989). They divide and grow in an undifferentiated state in culture but when they are re-introduced into blastocysts they are able to colonize the early embryo and can contribute to somatic tissues and germ cells, resulting in chimaeric mice (Bradley *et al.*, 1984). DNA can be introduced into ES cells either by microinjection or by electroporation (Capecchi, 1989). Manipulated ES cells provide a vehicle in which genetic modifications can be introduced from tissue culture to the mouse germline. In addition to the introduction of exogenous genes, recent advances using homologous recombination and gene targeting strategies have allowed the deletion or alteration of endogenous genes in ES cells (Capecchi, 1989; Thomas, 1994). Gene targeting by homologous recombination involves the replacement of normal genes with transgenes coding for mutant or null alleles. The vectors carrying the desired mutation have gene sequences homologous to the endogenous sequences with which they can recombine. Vectors which have successfully integrated at homologous sites, as opposed to at random sites, can then be selected and enriched for (Capecchi, 1989; Hasty and Bradley, 1995). The targeted ES cells are introduced into the mouse blastocyst. Transgenic knockout mice, also referred to as nulls, are generated by cross-breeding to produce animals homozygous for the deleted or disrupted gene.

1.7.2 Targeting and Expression of Transgenic Sequences.

Expression of a transgene can be directed to a range of different tissues or cell types by fusing the gene sequence of interest with particular promoters and enhancers (Palmiter and Brinster, 1985; Jaenisch, 1988). Depending on the nature of the regulatory

elements, it is possible to achieve broad spectrum or tissue restricted expression. Brinster *et al.* (1981) were the first to confirm transgene expression in mice microinjected with a fusion plasmid of herpes thymidine kinase linked to the regulatory region of the mouse metallothionein-1 gene. These investigators found high levels of thymidine kinase activity in the liver and kidney of the transgenic mice. However, it was noted that transgene expression was unpredictable, and that even although the transgene had integrated into the mouse DNA, this did not always result in gene expression in somatic tissues (Brinster *et al.*, 1981).

The level of transgene expression is generally unpredictable and is usually independent of transgene copy number. It has also been shown that expression of a transgene need not be restricted to the targeted tissue. This was observed for example with v-Ha-*ras* under the control of the MMTV long terminal repeat where expression of the transgene occurred in numerous tissues (Tremblay *et al.*, 1989). A number of factors, in addition to the promoter and enhancer sequences, can influence transgene expression (Jaenisch, 1988). It seems that the site of chromosomal integration is of particular importance (Al-Shawi *et al.*, 1990). Locus control regions in the transgenic construct can override the dependence on the integration site (Grosveld *et al.*, 1987). This was observed with a human CD2 transgene (Lang *et al.*, 1988). Expression of this transgene was restricted to thymocytes, T cells and megakaryocytes. The expression of the CD2 transgene was dependent on the copy number of genes which had integrated into the genome and apparently independent on the site of integration. The presence of locus control regions associated with the CD2 gene accounted for the copy-dependent, position-independent expression pattern of this transgene (Greaves *et al.*, 1989).

1.7.3 Transgenic Oncogene Mice.

The inaugural transgenic cancer model was described by Brinster and co-workers (1984) who demonstrated that transgenic mice expressing SV40 T-antigen genes developed tumours within the choroid plexus. Shortly thereafter, Stewart *et al.* (1984a) showed that the c-*myc* oncogene targeted to mammary gland tissue elicited mammary adenocarcinomas. There now exist transgenic models for many classes of oncogene,

including transcription factors, cell survival genes, growth factors, growth factor receptors and viral genes (Adams and Cory, 1991b; Hanahan, 1988; Pattengale, *et al.*, 1989).

These transgenic models have led to a greater understanding of the mechanisms by which oncogenes transform cells. The effect of an oncogene on a particular tissue can be ascertained by targeting expression of the transgene to a restricted cell lineage. For example, the c-*neu* oncogene linked to the MMTV promoter can induce mammary adenocarcinomas (Muller *et al.*, 1988). Likewise, mammary carcinomas are induced by MMTV-*ras* and MMTV-*myc* (Stewart *et al.*, 1984a; Sinn *et al.*, 1987). The spectrum of tissues that are susceptible to a particular transforming gene can also be delineated. The SV40 T antigen, *ras* oncogene and *myc* oncogene can induce tumours in a number of different cell types (Hanahan, 1988; Adams and Cory, 1991b). In contrast, widely expressed *fos* and *jun* transgenes appeared to induce cell-type specific tumours (Ruther *et al.*, 1987; Schuh, *et al.*, 1990). Therefore, the transforming capabilities of oncogenes can be wide ranging or cell type specific. Although the *myc* oncogene can transform many tissue types, it does not transform all cell types. This was demonstrated in one line of MMTV-*myc* mice where, although aberrant expression of c-*myc* was recorded in a range of tissues including the pancreas, lung and salivary gland, these tissue types did not appear to be as susceptible to transformation (Leder *et al.*, 1986).

1.7.4 Transgenic Models In Lymphomagenesis.

1.7.4.1 The *myc* oncogenes.

To model the chromosomal rearrangement which occurs in Burkitt's lymphoma, the immunoglobulin heavy chain enhancer (Eμ) was fused to the c-*myc* oncogene (Adams *et al.*, 1985). A high proportion of Eμ-*myc* mice were found to develop clonal tumours of pre-B or B cell origin similar to those observed in the human malignancy. By 4 months of age, 94% of Eμ-*myc* mice, from several independent lines, died of an aggressive lymphoma. The clonality and spontaneous nature of these tumours suggested that secondary events were required for full tumourigenesis. Deregulated *myc* expression

induced a pre-lymphomatous hyperplasia in the Eμ-*myc* mice (Langdon *et al.*, 1986) which presented as an expanded population of non-malignant, proliferating pre-B cells in the spleen and bone marrow (Alexander *et al.*, 1987). The *myc* expressing cells were found to be in cycle (Langdon *et al.*, 1986), promoting cell division over differentiation. It has been suggested that the transgene serves to predispose the tissue to further changes by creating a large population of cells which are then susceptible to secondary mutations (Adams and Cory, 1992).

Expression of the c-*myc* oncogene has also been targeted to the T-cell lineage (Spanopoulou *et al.*, 1989; Stewart *et al.*, 1993). The murine c-*myc* gene was inserted into the mouse Thy-1 transcriptional unit to direct expression of the gene to the thymus (Spanopoulou *et al.*, 1989). Thymic tumours arose in the *Thy-myc* mice between 9 and 14 weeks of age. The thymomas consisted of proliferating thymocytes and unexpectedly, expanded populations of epithelial cells. Histological diagnosis confirmed that lymphoid and stromal cell populations had expanded. Both cell types were found to establish in long term culture suggesting that they were indeed transformed. Large blast cells with the potential to metastasize had populated the thymus in these mice. The clonal tumours were mostly Thy-1+, CD4+CD8+ with rearranged T cell receptor (TcR) β-chain, suggesting that transformation had occurred in immature thymocytes. Transgenic mice carrying the human c-*myc* gene under the regulation of the CD2 locus control region were also predisposed to thymic lymphoma development (Stewart *et al.*, 1993). These mice developed clonal CD3+, CD4+/-, CD8+ T-cell tumours. Lymphoid tumours also developed in an MMTV-*myc* transgenic line which had widespread c-*myc* expression (Leder *et al.*, 1986). This observation confirms the potency of the c-*myc* oncogene in transforming lymphoid cells, especially as tumours did not arise in all transgene expressing tissues of the MMTV-*myc* line.

Transgenic mice with expression of the N-*myc* gene directed to the lymphoid compartment with the Eμ enhancer developed pre-B and B cell lymphomas with a preneoplastic stage similar to that observed with Eμ-*myc* mice (reviewed by Morgenbesser and DePinho, 1994). Eμ-L-*myc* mice demonstrated pre-tumourous disturbances in the thymus and spleen with a predisposition to developing T-lymphoid

tumours (Moroy *et al.*, 1990). The preferential expression of Eμ-L-*myc* in T cells was surprising considering the usual targeting of *myc* genes with the Eμ enhancer to the B-cell compartment and may be due to elements within the L-*myc* gene (Moroy *et al.*, 1990).

1.7.4.2 The *bcl*-2 oncogene.

The chromosomal translocation associated with human follicular lymphoma was recreated in the *bcl*-2-Ig transgenic mouse (McDonnell, 1993b). This transgene was derived from the fusion of the *bcl*-2 gene with the immunoglobulin (Ig) heavy chain locus, leading to deregulated expression of the *bcl*-2 oncogene in lymphoid tissue (McDonnell *et al.*, 1989). Lymphoid hyperplasia, with polyclonal expansion of non-dividing B cells, occurred in the spleen of *bcl*-2-Ig transgenic mice between 8 and 19 weeks of age (McDonnell *et al.*, 1989; McDonnell *et al.*, 1990). This non-cycling augmentation of lymphoid cells was consistent with the observed function of *bcl*-2 in enhancing cellular survival by inhibiting cell death (Hockenbery *et al.*, 1990). The benign, follicular hyperplasia of *bcl*-2-Ig mice progressed to high-grade malignant lymphoma after a latency of approximately 15 months (McDonnell and Korsmeyer, 1991). The need for secondary changes to proceed from the *bcl*-2-Ig initiated B-cell accumulation to clonally transformed tissue was implicated by the long latency of the lymphomas. Indeed, spontaneous c-*myc* rearrangements were detected in half of the malignant tumours but not in any of the *bcl*-2 over-expressing preneoplastic cells (McDonnell and Korsmeyer, 1991).

The human *bcl*-2 gene was targeted to the T-cell lineage using the lymphoid-specific tyrosine kinase, *lck* promoter. Over a period of 24 months, 26% of mice carrying the *lck-bcl*-2 transgene presented with large-cell lymphoma at a mean age of 18 months (Linette *et al.*, 1995). T-cell origin of the lymphomas was confirmed by flow cytometry analysis. The tumour cell population was predominantly CD4+CD8- which is compatible with a mature peripheral T-cell phenotype. The long latency of these tumours is similar to that seen with the *bcl*-2-Ig follicular lymphomas, but unlike the *bcl*-2-Ig transgenic

mice (McDonnell and Korsmeyer, 1991), *lck-bcl*-2 mice were not found to harbour spontaneous *myc* rearrangements.

1.7.4.3 *pim*-1 and other oncogenes.

The *pim*-1 gene was first discovered as a common viral integration site in T-cell lymphomas induced by Moloney murine leukaemia virus, MoMuLV (Cuypers *et al.*, 1984). Eμ-*pim*-1 transgenic mice developed a low incidence (5-7%) of spontaneous T cell lymphomas by 7 months of age, with no apparent preneoplastic abnormalities (van Lohuizen *et al.*, 1989). Although tumour frequency was low in Eμ-*pim*-1 transgenic mice, these mice were highly susceptible to T-lymphomagenesis induced by MoMuLV. The true oncogenic potential of *pim-1* was recognized by its ability to collaborate with other oncogenes in B- and T-cell tumours.

A number of other known oncogenes, including SV40 T antigen, H-*ras*, *bcr-abl* and *neu*, have been shown to induce lymphomagenesis in transgenic mice (reviewed by Adams and Cory, 1991a). Transgenic mice expressing the transcription factor *rbtn*-2 in the T-cell compartment confirmed the involvement of this gene in T-cell lymphoma (Larson *et al.*, 1994). CD2-*rbtn*-2 mice were predisposed to lymphoblastic lymphoma with a latency of between 5 and 15 months. Constitutive expression of the growth factor Il-9 in a transgenic model has also been observed to induce a low incidence of clonal T-cell tumours (Renauld *et al.*, 1994).

1.7.5 Transgenic Models Of Oncogene Synergy.

It is now widely accepted that tumourigenesis occurs as a multistep process (Bishop, 1991; Weinberg, 1989). Studies using transgenic mice further validate this hypothesis. In general, transgenic mice carrying a single oncogene develop tumours which arise sporadically after a considerable latency period. Tumours do not ensue from every transgene expressing cell but rather are usually clonal in origin. Moreover, a proliferative hyperplasia is often evident prior to neoplastic transformation (Hanahan, 1988), as has been observed with transgenic animals expressing *myc* and *bcl*-2

oncogenes (Langdon *et al.*, 1986; McDonnell *et al.*, 1990). Additional events are therefore necessary for the complete progression to malignancy. Naturally occurring spontaneous mutations have been identified in some transgenic tumours, for example in *bcl*-2-Ig follicular lymphomas where rearrangement of the *c-myc* gene occurred in at least half of the tumours (McDonnell and Korsmeyer, 1991). Rearrangements of the c-*myc* gene have also been observed in a high proportion of plasmacytomas arising in Eμ-v-*abl* mice (Rosenbaum *et al.*, 1990).

The predisposed state of the transgenic mouse to neoplasia provides an ideal model to investigate secondary genetic changes. These events can be experimentally introduced by the retroviral introduction of genes, by proviral insertional mutagenesis, or by crossing transgenic lines containing oncogenes directed to the same tissue (Berns *et al.*, 1989; Christofori and Hanahan, 1994). Using retroviral vectors, Langdon *et al.* (1989) introduced v-H-*ras* and v-*raf* oncogenes into the germline of Eμ-*myc* transgenic mice to show that both of these genes could collaborate with *myc* in transforming B cells. Alexander *et al.* (1989) used *in vitro* experiments to demonstrate that Eμ-*myc* bone marrow cells infected with retroviruses bearing the v-H-*ras* and v-*raf* oncogenes generated a rapid polyclonal expansion of pre-B cells which grew as tumours in nude mice. Conversely, v-*abl* did not collaborate with constitutive *myc* expression to promote accelerated lymphomagenesis either *in vivo* or *in vitro* (Langdon *et al.*, 1989; Alexander *et al.*, 1989).

1.7.5.1 Proviral insertional mutagenesis.

Proviral insertional mutagenesis has also been used to identify collaborating oncogenes in transgenic mice. Slow-transforming retroviruses, such as MoMuLV, do not contain their own oncogene and so rely on activation, or inactivation, of host genes in order to transform the cell. To achieve this, the retrovirus integrates into the host genome and activates cellular proto-oncogenes (reviewed by Kung *et al.*, 1991). Proviral insertion is a random process, however in many tumour cells integration apparently occurs at nonrandom common integration loci (reviewed by Tsichlis and Lazo, 1991). This is probably due to selection of the cells which carry integrations resulting in a growth

advantage. Van Lohuizen *et al.* were the first to use this procedure to look for collaborating oncogenes in transgenic mice. These authors demonstrated that insertion of the provirus acted as a tag by which to identify collaborating host genes (van Lohuizen *et al.*, 1989).

Proviral insertion has been used with some success to identify cooperating oncogenes in Eμ-*myc* transgenic mice. Two independent laboratories observed that infection of Eμ-*myc* mice with MoMuLV accelerated the rate of B cell lymphoma development indicating the action of a cooperating event(s). Several common proviral integration sites in these tumours were identified. Insertions at the *pim*-1 locus occurred in 19 - 35% of MoMuLV induced Eμ-*myc* tumours (van Lohuizen *et al.*, 1991; Haupt *et al.*, 1991) implicating *pim*-1 as a probable *myc* collaborating gene. In addition, insertions at novel loci, *bmi*-1, *emi*-1 and *pal*-1 were identified. No rearrangements of the *abl*, *raf*, *bcl*-2, *myb*, *ras*, *cbl*, or *rel* proto-oncogenes were identified by proviral insertion in these studies. Frequent proviral insertions at the *bmi*-1 locus were observed, with 35 - 47% of the Eμ-*myc* tumours carrying this integration (van Lohuizen *et al.*, 1991; Haupt *et al.*, 1991). Integrations at this locus had previously been unknown and it was suggested that the *bmi*-1 gene may play an exclusive role in B cell transformation (van Lohuizen *et al.*, 1991). The oncogenic potential of this gene was confirmed in Eμ-*bmi*-1 transgenic mice (Haupt *et al.*, 1993). By crossing the Eμ-*bmi*-1 transgenic mice with Eμ-*myc* mice it was established that the *bmi*-1 transgene markedly accelerated the onset of pre-B and B-cell lymphomas from greater than 10 weeks to less than 5 weeks of age, confirming the collaboration between these genes.

Some of the proviral insertions in the Eμ-*myc* mice were found to co-exist within the same tumour. Van Lohuizen *et al.* found co-existing *pim*-1 and *bmi*-1 mutations in 3 tumours, and co-existing mutations in *pal-1* and *pim*-1 in 4 other tumours (van Lohuizen *et al.*, 1991). Insertions at both *pim*-1 and *bmi*-1 were also observed by Haupt *et al.* (1991), occurring in 2 out of 30 tumours. This group also found co-existing insertions in both *bmi*-1 and *emi*-1 (2/36) and in *emi*-1 and *pim*-1 (1/30). These results could infer the existence of different complementation groups. As *myc*, *pim*-1 and *bmi*-1 can effectively cooperate in tumourigenesis, these oncogenes probably belong to different

complementation groups, contributing separate functions to the neoplastic process. That co-existing mutations in *bmi*-1 and *pal*-1 were not observed suggests that these oncogenes could belong to the same complementation group. A study of feline lymphoid tumours similarly reported the existence of complementary and non-complementary oncogenes (Tsatsanis *et al.*, 1994).

While *pim*-1 insertions were common in B-cell tumours induced by MoMuLV infection of Eμ-*myc* mice, reciprocal experiments demonstrated that *c-myc* and N-*myc* insertions consistently arose in T-cell lymphomas of Eμ-*pim*-1 transgenic mice infected with MoMuLV (van Lohuizen *et al.*, 1989). These results show that the collaboration between the *myc* and *pim*-1 oncogenes can occur in both the B- and T-cell lineages.

1.7.5.2 Transgenic crosses.

A further indication of the potency of the collaboration between the Eμ-*myc* and Eμ-*pim*-1 genes has been observed in crossing these two transgenic lines. Mice carrying both the Eμ-*myc* and Eμ-*pim*-1 transgenes exhibited pre-B-cell lymphoma *in utero* and did not survive to birth (Verbeek *et al.*, 1991). Similarly, double transgenic mice with both the Eμ-*pim*-1 gene and the Eμ-N- or Eμ-L-*myc* gene generated lymphoid tumours at an accelerated rate compared to those of either transgene alone (Moroy *et al.*, 1991). That the Eμ-*pim*-1 mice crossed with Eμ-N and L-*myc* mice survived to after birth, whereas Eμ-*pim*-1/Eμ-c-*myc* mice died *in utero* may reflect the weaker oncogenic action of N-*myc* and L-*myc*.

Dramatic acceleration in tumour development was also observed when mice expressing MMTV-H-*ras* were crossed with MMTV-*myc* mice to generate double transgenic mice (Sinn *et al.*, 1987). Hybrid mice accentuated the tumour phenotype of the single transgenic animals, developing primarily mammary adenocarcinomas but with a significantly reduced latency. Likewise, an Eμ-*myc*/Eμ-*bcl*-2 transgenic cross resulted in a rapid onset of lymphomagenesis (Strasser *et al.*, 1990). These double transgenic mice developed disseminated malignant lymphoma at 5 - 6 weeks, considerably faster than the Eμ-*myc* and Eμ-SV-*bcl*-2 parental groups. The mechanism of cooperation between

these two events has been ascribed to *bcl*-2 suppression of c-*myc* associated apoptosis (Marin *et al.*, 1995). A transgenic cross has also demonstrated collaboration between the *pim*-1 and *bcl*-2 oncogenes (Acton *et al.*, 1992). This collaboration was relatively weak with an average latency period of 30 weeks. Retroviral insertion of Eμ-*pim*-1/*bcl*-2-Ig hybrid mice with MoMuLV indicated common integration sites at N-*myc*, c-*myc* and *pal*-1. It is possible that activation of these genes acted as a tertiary event in the stepwise progression of T-cell lymphoma.

Although two and three genetic collaborations have been identified in these transgenic models, it has been intimated that these events alone are still insufficient for total neoplastic transformation. Even the strong synergy between the *pim*-1 and c-*myc* oncogenes requires further events as evident from transplantation experiments (Verbeek *et al.*, 1991). One exception to the multistep transgenic model has been observed with the c-*neu* oncogene (homologue of *erbB*-2) linked to the MMTV promoter. Synchronous, polyclonal adenocarcinomas in all mammary glands arose in the MMTV-c-*neu* mice at a young age (Muller *et al.*, 1988), suggesting that this gene alone was sufficient to induce tumours in mammary epithelium. This may be due to the particular combination of oncogene and tissue type as expression of *c-neu* in the parotid gland and epididymis did not initiate full malignant transformation.

1.7.6 Transgenic Knockout Mice.

The deletion and disruption of endogenous mouse genes by gene targeting and homologous recombination has made it possible to depict the normal function of a number of cellular genes and proto-oncogenes by creating null mice (recently listed in Brandon *et al.*, 1995). The role of the *bcl*-2 gene as an inhibitor of apoptosis was confirmed in *bcl*-2 null mice (Veis *et al.*, 1993). These mice exhibited several abnormalities including fulminant apoptosis of the thymus and spleen. A c-*myc* null mutation demonstrated the need for a functional c-*myc* gene during normal embryonic development when this mutation resulted in embryonic lethality (Davis *et al.*, 1993). Null transgenics have been particularly informative in the case of tumour suppressor genes, where in conventional transgenics the desired loss of function phenotype could

not be simulated. As negative growth regulators, it was necessary to inactivate or delete both alleles of these genes.

1.7.6.1 The *RB* knockout mouse.

To investigate the role of the *RB* gene in mammalian development and tumourigenesis, several laboratories generated *RB* knockout mice using homologous recombination (Lee *et al.*, 1992; Jacks *et al.*, 1992; Clarke *et al.*, 1992). Mice homozygous for this mutated allele, which produces a non-functional protein, were non-viable and died between days 14 and 15 of gestation. Severe abnormalities of the nervous and haematopoietic systems occurred in these embryos from day 10 of gestation due to massive cell death (Lee *et al.*, 1992; Jacks *et al.*, 1992; Clarke *et al.*, 1992). Therefore, *RB* was shown to be essential for normal mouse development. The heterozygous phenotype of these *RB* knockout mice surprisingly showed no evidence of retinoblastoma or retinal abnormalities but did demonstrate a predisposition to pituitary tumours. These tumours were shown to have lost the remaining *RB* wild type allele providing formal evidence for the role of *RB* as a tumour suppressor.

1.7.6.2 The p53 knockout mouse.

Inactivation of the p53 tumour suppressor gene has been described in several different transgenic knockout models (Donehower *et al.*, 1992; Purdie *et al.*, 1994; Jacks *et al.*, 1994). Unlike the *RB* homozygous null mouse, p53 nullizygous mice were viable and appeared normal at birth. However, the p53 null mice were observed to spontaneously develop a wide range of tumour types between 3 and 10 months of age, with a predominance for T-cell lymphoma. Heterozygous p53 null mice were also found to be predisposed to tumour development, albeit with a longer latency and a significantly different tumour spectrum, sarcoma type tumours predominating. These tumours were often found to be associated with loss of the remaining wild type p53 allele (Harvey *et al.*, 1993a; Purdie *et al.*, 1994; Jacks *et al.*, 1994). These observations confirmed the role of p53 as a general tumour suppressor gene.

Studies employing p53 knockout animals were important in establishing the requirement for p53 in DNA-damaged induced apoptosis (Lowe *et al.*, 1993a; Clarke *et al.*, 1993). These groups independently observed that p53 null thymocytes were unable to elicit an apoptotic response to irradiation and etoposide but were susceptible to cell death on treatment with glucocorticoid. Furthermore, heterozygous null cells showed an intermediate susceptibility to radiation-induced apoptosis. The use of these tumour prone animals has also been applied to *in vivo* carcinogenesis studies. Harvey *et al.*, for example, demonstrated that p53 heterozygote mice were more susceptible to carcinogen-induced liver tumours than wild type mice when treated with dimethylnitrosamine (Harvey *et al.*, 1993a). Also, the initiation of skin papillomas following chemical induction was found to be independent of p53 loss, but malignant progression was greatly enhanced in the absence of functional p53 (Kemp *et al.*, 1993). As these examples demonstrate, the p53 null and heterozygote null mice have provided a valuable tool for studying the function and role of the p53 tumour suppressor gene. Many applications of this model are currently in use.

CHAPTER 2.

MATERIALS & METHODS.

2.1 ANIMALS.

All animal work was carried out under Home Office regulations.

2.1.1 Transgenic Mouse Stocks.

The generation of CD2-*myc* transgenic mice was carried out by Dr. E. Cameron and has been described previously (Stewart *et al.*, 1993). In order to target the c-*myc* gene to the T-cell lineage, the human c-*myc* locus (HindIII-EcoRI fragment in plasmid pMC41) was fused to a regulatory domain of the human CD2 locus (5.5 kb BamHI-Xba fragment) to construct the CD2-*myc* plasmid (Figure 2.1) as described in Stewart *et al.*, 1993. This work was carried out by Dr. M. Stewart. The linearized CD2-*myc* plasmid was then microinjected into C57Bl/6J x CBA/Ca F2 (B6/CBA) fertilized eggs according to standard protocols (Hogan *et al.*, 1986). Two transgenic lines were established, the CD2-*myc*-800 and the CD2-*myc*-900 lines. The CD2-*myc*-800 line (hereafter referred to as CD2-*myc*) was selected for use in this study and was maintained on an indiscriminate B6/CBA background.

Mice deficient for a functional p53 tumour suppressor gene were derived using homologous recombination in murine ES cells by L. Donehower and co-workers (1992). A null mutation with an inserted *pol*II-*neo* expression cassette and 106 bp deletion in exon 5 of the p53 gene was introduced into the germ line of 129/Sv ES cells. Chimaeric mice, generated by introducing the targeted ES cells into C57Bl/6 blastocysts, were crossed onto a C57Bl/6 background. These mice were subsequently bred onto the NIH strain (Kemp *et al.*, 1993). The genetic background of the mice used in the studies described here were an indeterminate mix of C57Bl/6, 129/Sv and NIH strains. The use of the p53-deficient mice (hereafter referred to as p53 null or $p53^{-/-}$) has been with the kind permission of Dr. Larry Donehower.

Breeding stocks of both the CD2-*myc* transgenic and p53 null mice were maintained with heterozygous animals. All experimental cohorts were established from these breeding stocks.

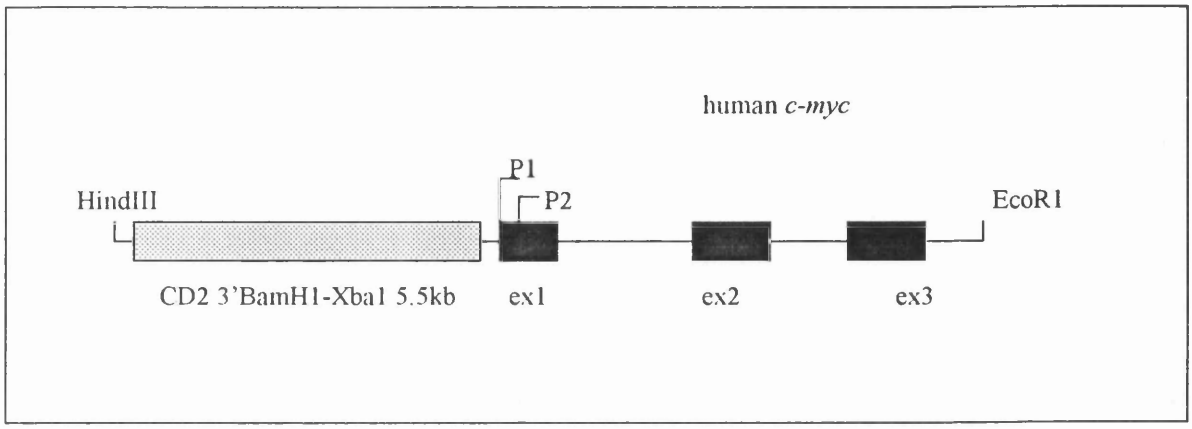

Figure 2.1. The CD2-*myc* Transgene.

The human *c-myc* locus was fused to a regulatory domain of the human CD2 locus at a PstI site 5' to the P1 initiation site as described in Stewart *et al.*, 1993.

2.1.2 Challenge of Mice with MoMuLV.

MoMuLV clone 1A supernatant was isolated from virus infected 3T3 cells (a gift of Dr. A. Berns) during the log phase of growth and filter sterilised through a 0.45μm filter (Gelmain Sciences) to remove cell debris. Aliquots of 1ml of filtered supernatant were frozen at -70°C until required. Neonatal animals were inoculated intraperitoneally with 0.1ml of supernatant, estimated at 10^4-10^5 infectious units of virus, within 24 hours of birth.

2.1.3 Mouse Genotype Analysis.

Positive transgenic animals were identified by screening tail DNA for the presence of the transgene/null mutation by Southern blot hybridisation.

Potential transgenic animals were weaned from the breeding stocks at 3-4 weeks of age and separated by sex. At 4-6 weeks of age, animals were anaesthetized by intraperitoneal administration of anaesthetic and a 1-2cm biopsy of tail taken. The tail wound was then cauterized. Anaesthetic was prepared from a combination of hypnorm (Janssen Animal Health) and hypnovel (Roche) in sterile water at a ratio of 1:1:8 and filter sterilized through a 0.45μm filter (Gelmain Sciences). Animals were tagged by an ear nicking identification scheme and allowed to recover. DNA extraction and Southern blot analysis on the tail tissue was carried out as described in section 2.3.

2.1.4 Clinical Examination and Post-mortem Investigation.

Experimental cohorts and breeding stocks of mice were maintained for defined periods of time and the health of animals checked at least five times weekly. The development of lymphoid neoplasia in the transgenic animals presented as cachexia and tachypnoea. Sarcomas and carcinomas, which often developed in p53 null mice, presented as visible swellings, paresis or cachexia depending on the anatomical site of the tumour. Animals were humanely sacrificed by cervical dislocation when clinical signs first became evident.

Post-mortem was routinely undertaken immediately after euthanasia, or as near to the time of death as possible, and the pathology recorded. Particular attention was paid to the lymphoid organs, specifically the thymus, spleen, mesenteric and subcutaneous lymph nodes. Evidence of metastasis to non-lymphoid organs was also noted. Total body weight of animals was recorded using a digital balance (Tefal). Thymic weight was measured using a scientific balance (Mettler). A range of tissue samples, both lymphoid and non-lymphoid, were taken at post-mortem. Tissues were frozen in cryotubes (Gibco BRL) in liquid nitrogen (BOC) for Southern and Northern analysis; fixed in 10% neutral buffered formalin for histopathological examination; and placed in RPMI medium (Gibco BRL) for cell biology experiments.

2.2 HISTOPATHOLOGY.

Material for histopathological examination was fixed in 10% neutral buffered formalin, wax/paraffin-embedded and stained with haematoxylin and eosin. Histopathological processing was carried out by Mr. I. MacMillan and staff. Histopathological examinations were carried out by Dr. S. Toth.

2.3 SOUTHERN HYBRIDISATION ANALYSIS.

DNA extraction and all Southern hybridisation analysis on mouse tissue was carried out by Dr. E. Baxter and Mrs. A. Terry. A brief description of this procedure is given below.

High molecular weight DNA was prepared from mouse tissues using guanidium chloride or Nucleon™ II kit (Scotlab). DNA digestion with restriction enzymes, separation by agarose gel electrophoresis, transfer to Hybond™ N membranes (Amersham International plc.), hybridisation and washing of blots were all carried out as described in Sambrook *et al.*, 1989. Probes were radiolabelled by nick translation or multiprime labelling as described in section 2.5.4. Transgene sequences were detected using a human c-*myc* exon 3 probe (1.38kb EcoR1/Cla1 fragment). The 260bp p53 exon 4 probe was generated from a plasmid subclone pLTRp53cG (Eliyahu *et al.*, 1985) by polymerase chain reaction (PCR) using oligo primers 5'-CCA TCA CCT CAC TGC ATG G-3' and 5'-CGT GCA CAT AAC AGA CTT GGC-3'.

2.4 PCR ANALYSIS OF GENOMIC DNA.

2.4.1 Isolation of Genomic DNA from Mouse Tissues and Cell Suspensions.

Genomic DNA was isolated from mouse tumour tissue and tumour cells using the Nucleon™ II method (Scotlab Bioscience). Frozen tissue was ground to a powder in liquid nitrogen using a chilled mortar and pestle (BDH). The tissue, or cell pellet, was resuspended in 2ml of Reagent B (400mM Tris-HCL at pH 8.0, 60mM EDTA, 150mM NaCL and 1% SDS) in a 15ml centrifuge tube by vortexing briefly. Deproteinisation was carried out by adding 500µl of Nucleon sodium perchlorate and rotary mixing for 15 minutes at room temperature. The sample was then incubated in a shaking water bath at 65°C for 25 minutes. DNA was extracted by adding 2ml of chloroform (BDH), which had been stored at -20°C. The sample was rotary mixed for 10 minutes at room temperature and then centrifuged at 3200 r.p.m. for 1 minute. 300µl of Nucleon silica suspension was added and the sample centrifuged at 4300 r.p.m. for 3 minutes. The DNA phase above the Nucleon silica suspension layer was transferred to a clean 15ml centrifuge tube and centrifuged briefly at 4200 r.p.m. to pellet residual Nucleon silica. The supernatant was carefully decanted into a clean 15ml centrifuge tube and two equal volumes of cold ethanol, at 4°C, added. The tube was inverted gently to precipitate the DNA. The precipitated DNA was spooled out into a 1.5ml eppendorf tube and left to dry. The DNA was resuspended in 200 - 400µl of ultra pure water by leaving the sample overnight at room temperature. The DNA concentration was determined by measuring the optical density of the sample at 260nm. The DNA sample was stored in an eppendorf tube at 4°C.

2.4.2 PCR Determination of p53 Status.

All PCR analysis was carried out by Mr. C. Johnston and Ms. J. Irvine. The following information was kindly provided by Mr. C. Johnston. Amplification of genomic DNA was carried out in a 50µl reaction mix which consisted of 1µg of DNA, 2 units of Taq polymerase (Perkin Elmer), 200µM of each deoxynucleoside triphosphate, primer

sequences to a final concentration of 0.5μM in 10mM Tris (pH 8.4), 50mM KCl and 1.5mM $MgCl_2$. Thermal cycling was performed on a Perkin Elmer 9600 with an initial denaturation of 5 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C, with a final extension phase of 7 minutes at 72°C. Primer sequences used to detect the wild type p53 allele were directed against regions of intron 4 (WP53) 5'-GTG TTT CAT TAG TTC CCC AC-3' and exon 5 (UP3) 5'-ATG GTG GGG GCA GCG TCT CA-3'. The p53 null allele was detected using primer sequences NP5; 5'-CGG TCT TGT CGA TCA GGA TG-3' and UP3. Primers specific for the wild type allele generated a 242 bp product, while primers specific for the p53 null allele generated a 470 bp product. The PCR products were subjected to electrophoresis, either on an 8% acrylamide/Bis-acrylamide gel at 120V for 1 hour in TBE buffer, or on a 1.5% agarose gel at 100V for 3 hours in TBE buffer. Gels were stained with ethidium bromide and photographed under ultraviolet transillumination.

2.5 NORTHERN HYBRIDISATION ANALYSIS.

2.5.1 Buffers.

RNA Loading Buffer

50% of formamide (Gibco BRL), 2.2M of formaldehyde solution (BDH) and 1x MOPS buffer (see below) were added to 1.17ml ultra pure water and stored at -20°C.

10 x MOPS Running Buffer

200mM of MOPS (Sigma), 50mM of sodium acetate (Sigma) and 1mM of EDTA (Sigma) were made to 1 litre ultra pure water, adjusted to pH 7.0 using 10M sodium hydroxide (Sigma) and stored at room temperature in a dark bottle.

24 x SSC

1000g of sodium chloride (Sigma) and 500g of citric acid (Sigma) were dissolved in 4.7 litres of deionised water and stored at room temperature.

10 x SSC

416ml of 24 x SSC was made to 1 litre in deionised water and stored at room temperature.

2.5.2 RNA Extraction.

Total cellular RNA was prepared from mouse tissues using the RNAzol^{TM}B method (Biogenesis, Bournemouth). Frozen tissue was ground to a powder in liquid nitrogen using a chilled mortar and pestle (BDH), and resuspended in 2ml per 100mg of tissue, in cold RNAzol^{TM}B in a 15ml centrifuge tube. One tenth volume of chloroform (BDH) was added to the homogenate, the sample shaken vigorously for 20 seconds and left on ice for 5 minutes. Samples were then centrifuged at 13000 r.p.m. at 4°C for 10 minutes. The aqueous phase was transferred to 1.5ml eppendorf tubes and an equal volume of isopropanol (Sigma) added to precipitate the RNA. Samples were stored at 4°C for at least 15 minutes then centrifuged at 13000 r.p.m. at 4°C for 15 minutes and the supernatant removed. The RNA pellet was washed with 1ml of 75% ethanol by vortexing and subsequent centrifugation at 13000 r.p.m. for 5 minutes at 4°C. The ethanol supernatant was discarded and the pellet resuspended in 40µl of 1mM EDTA (Sigma) which had been treated with DEPC (Sigma). The RNA concentration was determined by measuring the optical density of the sample at 260nm. The RNA sample was stored in an eppendorf tube at -70°C.

2.5.3 RNA Blotting.

The RNA sample, prepared as described above, was dissolved in ultra pure water to a concentration of 10-15µg in 10µl. To this, 17µl of RNA loading buffer was added. The sample was then heated to 65°C for 15 minutes and placed on ice for 5 minutes. 3µl of loading dye, bromophenol blue (Sigma) in water, was added and the sample pulse centrifuged to collect the contents.

The RNA was separated on a 1% agarose gel containing 2.2M analar formaldehyde (BDH). For a 15cm x 15cm gel tray, 20ml of 10 x MOPS buffer and 33ml of

formaldehyde (BDH) were heated in a 37°C water bath and added to 2g agarose (Gibco BRL) melted in 147ml deionised water. The gel was left to set at room temperature. Electrophoresis was carried out overnight in 1 x MOPS running buffer at 20V. The RNA gel was subsequently washed on a shaking platform in deionised water for 5 hours with at least three changes, stained with 50μg/100ml ethidium bromide for 30 minutes and destained with deionised water for 30 minutes. The gel was viewed by ultraviolet transillumination and photographed to confirm the presence of RNA in the lanes.

The RNA was transferred to Hybond™ N membrane (Amersham International plc.) using upwards transfer in 10 x SSC as described by Sambrook *et al.*, 1989. The RNA was immobilized to the nylon membrane by crosslinking using a UV Stratalinker 1800 (Stratagene).

2.5.4 Radiolabelled Probes.

Detection of the CD2-*myc* transgene was with a human c-*myc* exon 3 probe generated from a 1.38kb EcoRI/ClaI fragment prepared by Ms. J. Irvine. As a control, blots were analysed for the glyceraldehyde phosphate dehydrogenase (GAPDH) gene. This probe was generated from a 720bp fragment of rat GAPDH gene as amplified by PCR from rat cDNA by R. Hennigan.

Using a multiprime DNA labelling kit (Amersham International plc), c-*myc* and GAPDH fragments were radiolabelled by random priming to generate dsDNA probes. 50-100ng/μl of fragment was made to 25μl with ultra pure water, boiled for 5 minutes and chilled on ice. 5μl of primer, 10μl of labelling buffer and 2μl of klenow enzyme were added, pulse centrifuged at 13000 r.p.m. and chilled on ice. 8μl of [α32P]dCTP (>3000 Ci/mmol, Amersham International plc) was added and left overnight at room temperature. The next morning the labelled fragment was eluted through a nick column (Pharmacia) and stored at -20°C for up to 2 weeks.

2.5.5 Hybridisation of RNA Blots.

Before hybridisation, RNA blots were pre-incubated with 10-20ml of hybridisation solution in a roller bottle (Techne) at 65 - 68°C. Radiolabelled dsDNA probe was boiled for 5 minutes and chilled on ice prior to addition of 100-150µl to the pre-hybridised blots. The blots were hybridised for 2-3 hours at 65°C using QuickHyb™ (Stratagene) or RapidHyb™ (Amersham International plc) hybridisation solutions as per suppliers instructions. When using QuickHyb™, 500µl of Genebloc (Immunogen International) was also added. Blots were washed twice at 65°C with 2 x SSC; 0.1% SDS (Sigma) for 15 minutes and twice at 65°C with 0.2 x SSC; 0.1% SDS for 15 minutes and set up for autoradiography with Hyperfilm™-MP (Amersham International plc.).

2.6 Tissue Culture Techniques.

2.6.1 Media.

Complete RPMI

Media was prepared using aseptic techniques. RPMI 1640 medium (Gibco BRL) was supplemented with 10% heat inactivated foetal calf serum, 100U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine (all from Gibco BRL) and 5 x 10^{-5} M 2-mercaptoethanol (BDH).

Cell Freeze Down Medium

10% dimethyl sulphoxide (Merck Ltd.) and 20% foetal calf serum (Gibco BRL) was made up in RPMI 1640 medium and filter sterilized through a 0.45µm filter (Gelmain Sciences) in aseptic conditions.

2.6.2 Single Cell Tumour Preparation.

Tumour/thymus tissue was disaggregated aseptically in complete RPMI medium using sterile scalpel blades (Fisons Scientific Equipment) in a 60mm tissue culture petri dish

(Gibco BRL). Lymphocytes were isolated on a Ficoll-Paque (Pharmacia) density gradient at 3000 r.p.m. for 10 minutes in a 15ml centrifuge tube (Falcon). The interphase layer containing the live lymphocytes was washed in 10ml complete RPMI medium and centrifuged at 1500 r.p.m. for 5 minutes. The supernatant was discarded and the cells resuspended in 5-10ml of complete RPMI medium. A viable cell count was carried out either on a Coulter Counter (model ZM, Coulter Electronics Ltd.) or by trypan blue (Gibco BRL) exclusion on a haemocytometer.

Cells were frozen to -100°C in 1.5ml Nunc cryotubes (Gibco BRL) at a concentration of 5-10 x 10^6 cells/ml in freeze down medium using a controlled rate freezer (Planar Products Ltd., model Kyro 10) and stored in liquid nitrogen (BOC).

2.6.3 Tumour Cell Line Establishment.

Tumour cells were prepared as in section 2.6.2. Cells at a concentration of 2.5 x 10^6 cells/ml in complete RPMI medium were cultured in 25cm^2 or 80cm^2 tissue culture flasks (Gibco BRL) at 37°C in an atmosphere of 5% CO_2 in air. Cell cultures were passaged every 3-4 days into fresh medium and maintained at a density of 5 x 10^5 - 5 x 10^6 cells/ml. Cell lines established for a time greater than 6 months were frozen to -100°C as described above.

2.7 IMMUNOPHENOTYPE ANALYSIS.

Single cell lymphocyte suspensions were prepared as described in section 2.6.2 and washed in cold phosphate-buffered saline (PBS, Gibco BRL) containing 0.1% BSA (Sigma) and 0.01% sodium azide (BDH). Cells at 1-2 x 10^6 per ml were centrifuged at 7000 r.p.m. for 3 minutes and the supernatant discarded. The cells were labelled by resuspending them with rat monoclonal anti-mouse antibody (Table 2.1) for 30 minutes at 4°C. 1-2μg of antibody was used to label 1-2 x 10^6 cells. Samples were centrifuged at 7000 r.p.m. for 3 minutes and washed with 200μl of cold PBS as described above. Two further rounds of this washing procedure were carried out. Indirect labelling with anti-

mouse CD3-biotin conjugate (Serotec) required a second 30 minute incubation with streptavidin allophycocyanin (Cambridge Bioscience) followed by three washes in cold PBS as described. Cells were resuspended in 500µl of cold PBS, containing 0.1% BSA and 0.01% sodium azide, in sterile test tubes (Sarstedt) and analysed on a Coulter Epics Elite using the manufacturers recommended protocol. For each fluorochrome used, irrelevant isotype matched antibody controls were used to set a region to exclude background staining. Fluorescence above this threshold was considered as positive fluorescence.

Table 2.1 Rat Monoclonal Antibodies to Mouse Surface Antigens

Antibody	Clone	Isotype	(Supplier)
anti-mouse CD8a FITC conjugate	53-6.7	Rat IgG2a	(Gibco BRL/Sigma)
anti-mouse CD4 R-phycoerythrin conjugate	H129.19	Rat IgG2a	(Gibco BRL/Sigma)
anti-mouse CD3 Quantum Red conjugate	29B	Rat IgG2b	(Sigma)
anti-mouse CD45R FITC conjugate	RA3-6B2	Rat IgG2a	(Pharmingen/Sigma)
anti-mouse αβ TCR FITC conjugate	H57-597	Hamster IgG	(Pharmingen)
anti-mouse CD3 biotin conjugate	KT3	Rat IgG2a	(Serotec)

2.8 *IN SITU* DETECTION OF APOPTOSIS.

TdT-mediated, dUTP-biotin nick end labelling (TUNEL) allows the identification of apoptotic cells *in situ*. This method has been adapted from Gavrieli *et al.*, 1992.

2.8.1 Buffers.

TDT buffer

30mM Trizma base pH 7.2 (Sigma), 140mM sodium cacodylate (BDH) and 1mM cobalt chloride (Sigma) were made to 1 litre in ultra pure water and stored at room temperature.

TB buffer

300mM sodium chloride (Sigma) and 30mM sodium citrate (Sigma) were made to 1 litre in ultra pure water and stored at room temperature.

DN buffer

Solution A.

30mM Trizma base pH 7.2 (Sigma) and 140mM sodium cacodylate (BDH) were made to 1 litre in ultra pure water and stored at room temperature in a dark bottle.

Solution B.

4mM magnesium chloride (Sigma) was dissolved in Solution A and stored for not more than 1 week at 4°C in a dark bottle.

Solution C.

0.1mM DTT (Sigma) was made up in solution B (made fresh).

House detection diluent

1.21g Trizma base (Sigma), 2.92g sodium chloride (Sigma) and 0.2g magnesium chloride (Sigma) were dissolved in 80ml of ultra pure water and adjusted to pH 8.5. 50µl of Tween 20 (Sigma) was added for a final 100ml volume.

2.8.2 Tissue Preparation.

Tumour and thymus material was fixed in 10% neutral buffered formalin. The tissues were subsequently embedded in paraffin by Mr. I. MacMillan and staff. 1-2µm sections

were adhered to slides and deparaffinated at 70°C for 20-30 minutes then rinsed in a xylene substitute, histoclear (National Diagnostics), for 5 minutes. Alcohol hydration was carried out by washing the slides twice in 100% ethanol for 1 minute, once in 95% methylated spirit for 2 minutes and then held in distilled water.

2.8.3 Controls.

For each tissue sample a set of controls were prepared in addition to the test section. DNase treatment of the tissue section was used as a positive control. As a negative control, the section was incubated with TDT buffer instead of the TdT/dUTP reaction mix. The test section was treated with all incubations except the DNase reaction.

2.8.4 Nick End Labelling.

All incubations were carried out with 50-100µl of solution in a humidified chamber at room temperature except where stated. Sections were incubated with 20µg/ml proteinase K (Sigma) for 30 minutes, then washed 4 times in PBS (Sigma). Positive control slides were treated with DNase (20µg/ml in DN buffer solution C, Boehringer Mannheim) for 20 minutes and washed 4 times in PBS. Endogenous peroxidases were blocked by rinsing the sections in 3% hydrogen peroxide (Sigma) in methanol for 5 minutes. The sections were washed 4 times in PBS and immersed in TDT buffer for 2 minutes. TdT and biotin-16-dUTP (both Boehringer Mannheim) were diluted in TDT buffer to 1:100-1:200 (0.15-0.25U/µl) and 1:50-1:100 respectively. Sections, except negative controls, were incubated with the TdT/dUTP reaction mix for 60 minutes at 37°C. The reaction was stopped by rinsing the sections in TB buffer twice for 5 minutes. The samples were then washed in PBS 4 times. Incorporation of biotin-16-dUTP was detected by incubating the sections for 60 minutes with peroxidase conjugated streptavidin (Dako, Bucks) diluted 1:200 in house detection diluent. The sections were washed twice with PBS and stained with peroxidase substrate kit AEC (3-amino-9-ethyl-carbazole, Vector laboratories) for 15-30 minutes. After washing in water, the sections were counterstained with Mayers haematoxylin (Stevens, 1982) and mounted with supermount (Biomen Ltd.). Stained tissue sections were examined by light microscopy

(Leitz) and apoptosis quantified by determining the number of chromogen stained single cells and macrophages within a field at a magnification of x400. Approximately five fields were counted per slide.

CHAPTER 3.

ACCELERATED T-CELL LYMPHOMAGENESIS IN MICE WITH A CD2-*MYC* TRANSGENE AND P53 NULL MUTATION.

3.1 INTRODUCTION.

3.1.1 The CD2-*myc* Transgenic Mouse Model.

CD2-*myc* transgenic mice carry the c-*myc* oncogene under the regulation of the CD2 dominant control region (Greaves *et al.*, 1989) which targets expression to the T-cell lineage (Stewart *et al.*, 1993). Two transgenic lines carrying the CD2-*myc* construct were predisposed to a relatively low incidence (18% and 3%) of spontaneous T-cell lymphoma, occurring at between 3 and 12 months of age (Stewart *et al.*, 1993). High levels of transgene expression were detected in the thymic tumours of the CD2-*myc* mice, however, transgene mRNA was not found in normal transgenic thymus. The relatively low tumour incidence in these transgenic lines may be related to the lack of observable expression in the healthy tissues of these mice. Tumour incidence was however also found to be dependent on genetic background (Cameron *et al.*, 1996). The tumours arising in the CD2-*myc* transgenic mice were monoclonal or oligoclonal as determined by TcR and IgH gene rearrangements (Stewart *et al.*, 1993). Consistent with the multistep process of tumourigenesis (Weinberg, 1989; Bishop, 1991), the clonality, latency and random occurrence of the lymphomas in the CD2-*myc* mice implied that additional events were necessary to collaborate with the transgene in the pathogenesis of these tumours. The hypothesis that subsequent events were required for lymphomagenesis was supported by the marked reduction in latency and increase in tumour incidence observed in MoMuLV infected CD2-*myc* mice (Stewart *et al.*, 1993). To investigate which genes collaborated with the CD2-*myc* transgene in MoMuLV-induced tumours, a detailed analysis of proviral insertion sites has been carried out (Stewart *et al.*, 1996). These studies have identified infrequent insertions at *bmi*-1 (1/77), *pim*-1 (3/77) and *ahi*-1 (2/77). Significantly, a novel common insertion locus, *til*-1, was identified in 25 of 77 CD2-*myc* MoMuLV-induced lymphomas (Stewart *et al.*,

1996). The *til*-1 locus, which was localised to mouse chromosome 17, is thought to harbour a gene which specifically collaborates with c-*myc* in the development of T-cell lymphoma.

The CD2-*myc* transgenic mouse provides a useful model to investigate the genetic events which collaborate with deregulated c-*myc* in tumourigenesis. Oncogene collaboration can be effectively assessed by breeding together transgenic mice expressing different oncogenes (Berns *et al.*, 1989). The synergistic partnership of c-*myc* with each of the *bcl*-2, *pim*-1 and *bmi*-1 oncogenes in B-cell lymphomagenesis was demonstrated in this way (Strasser *et al.*, 1990; Verbeek *et al.*, 1991; Haupt *et al.*, 1993).

3.1.2 Potential Collaboration Between c-*myc* and p53 in Tumourigenesis.

Several points of reasoning implied that loss of function of the p53 tumour suppressor gene would co-operate with deregulated c-*myc* in tumour development. Firstly, many previous studies have shown that mutations of both the c-*myc* and p53 genes co-exist in the same human tumour or tumour cell line. Co-existing alterations of both p53 and c-*myc* have been in described in Burkitt's lymphoma and L3-type B-cell acute lymphoblastic leukaemia (Farrell *et al.*, 1991; Gaidano *et al.*, 1991), diffuse large cell lymphomas (Farrugia *et al.*, 1994), human oral cancer cell lines (Inagaki *et al.*, 1994) and in the clonal evolution of malignant lymphomas (Chang *et al.*, 1994). Furthermore, evidence has emerged to suggest that complementary genetic lesions which result in excessive cell proliferation and a block in cell death represents a potent combination for tumourigenesis (McDonnell, 1993a). This has been established, for example, in the dramatic acceleration of tumourigenesis in transgenic animals carrying both the Eμ-*myc* and Eμ-*bcl*-2 transgenes (Strasser *et al.*, 1990). Loss of p53 function has been shown to inhibit apoptosis under certain circumstances (Clarke *et al.*, 1993; Lowe *et al.*, 1993a; Lowe *et al.*, 1994a; Oren, 1994). Therefore, it could be hypothesised that p53 loss would provide a complementary lesion to the enhanced proliferative signal of activated c-*myc*.

3.1.3 Experimental Aims.

The aim of this study was to use a transgenic mouse model to investigate the combined effects of the CD2-*myc* lesion and loss of the p53 tumour suppressor gene product in tumour development. Mice which lack a functional p53 tumour suppressor gene (Donehower *et al.*, 1992) have proved to be an effective model for investigating the consequence of p53 loss *in vivo*. The predominance of T-cell lymphoma in these p53 null mice offered a distinct advantage with which to assess the potential synergy between deregulated c-*myc* and p53 loss in T-cell lymphomagenesis.

3.2 RESULTS.

3.2.1 Generation of Experimental Animals.

In order to assess the *in vivo* interaction between activated c-*myc* and p53 loss, hybrid transgenic mice with the CD2-*myc* transgene on a p53 null background were generated. This was achieved by crossing CD2-*myc* transgenic mice (Stewart *et al.*, 1993) with p53 null mice (Donehower *et al.*, 1992). As the p53 mutation was recessive to the wild type allele, it was necessary to create an F2 generation by subsequently breeding together animals heterozygous for both the CD2-*myc* transgene and p53 null gene. Mice heterozygous for both lesions were also backcrossed with p53 null mice. This breeding programme resulted in an experimental cohort of CD2-*myc* transgenic mice on homozygous null ($p53^{-/-}$/CD2-myc^{+}), heterozygous null ($p53^{+/-}$/CD2-myc^{+}) and wild type p53 ($p53^{+/+}$/CD2-myc^{+}) backgrounds. In addition, littermate controls of $p53^{-/-}$, $p53^{+/-}$ and $p53^{+/+}$ mice that did not carry the CD2-*myc* transgene were generated. These experimental groups, their genotypes and cohort numbers are given in Table 3.1. The various cohorts were subsequently monitored over a twelve month period.

Table 3.1 Experimental Mouse Cohorts Generated by Breeding Together p53 Null and CD2-*myc* Transgenic Mice.

Mouse Cohorts	p53 genotype status	CD2-*myc* genotype status	cohort size
$p53^{-/-}$/CD2-myc^{+}	homozygous null	positive	18
$p53^{+/-}$/CD2-myc^{+}	heterozygous null	positive	34
$p53^{+/+}$/CD2-myc^{+}	wild type	positive	10
$p53^{-/-}$	homozygous null	negative	11
$p53^{+/-}$	heterozygous null	negative	24
$p53^{+/+}$	wild type	negative	10

3.2.2 Lymphoma Development In Mice Carrying Both the CD2-*myc* Transgene and p53 Null Mutation.

Transgenic mice which carried both the CD2-*myc* transgene and homozygous p53 null mutation ($p53^{-/-}$/CD2-myc^{+}) were viable. As was predicted from the breeding programme, the expected numbers of $p53^{-/-}$/CD2-myc^{+} animals survived to birth (Table 3.1) indicating that the combination of these mutations did not result in pre-natal death. These mice appeared clinically normal from birth to post-weaning with no obvious evidence of developmental abnormalities.

In comparison to parental groups, the combination of CD2-*myc* and p53 null genotypes resulted in a marked acceleration in the rate of spontaneous lymphoma development (Figure 3.1). All of the $p53^{-/-}$/CD2-myc^{+} mice became terminally ill at between 60 and 86 days of age, much earlier and more synchronously than all other littermates. These mice were found to have developed lymphoma with 100% incidence (Table 3.2). The reduced latency of lymphomagenesis in the $p53^{-/-}$/CD2-myc^{+} mice was highly significant ($p < 0.001$, Mann-Whitney confidence test) when compared to each of the other groups in the study.

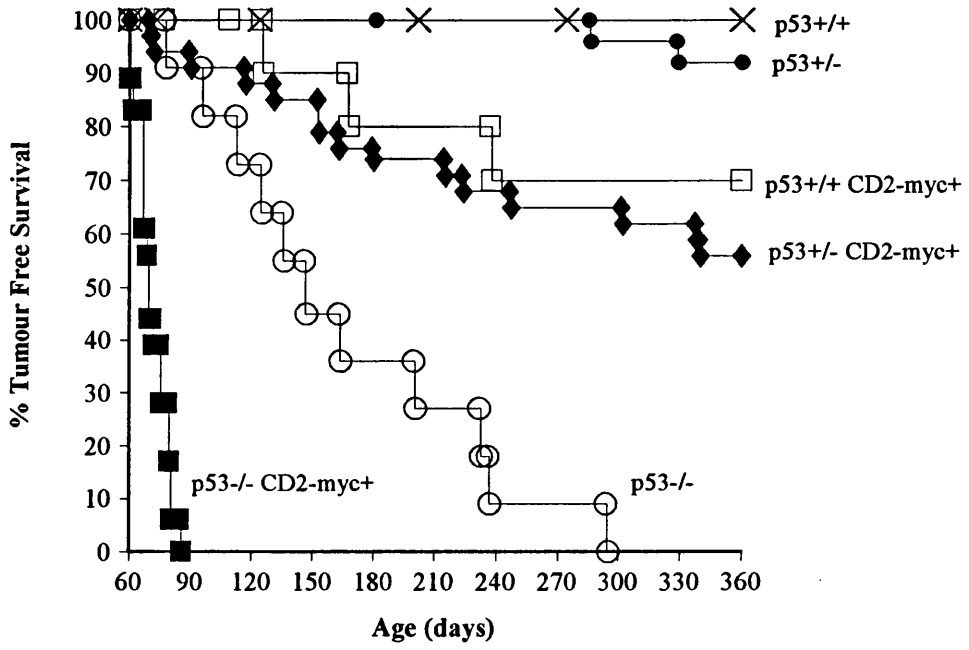

Figure 3.1. Tumour Free Survival of p53-/- CD2-myc+ Mice and Control Littermates.

The tumour free survival over a period of twelve months was observed for p53-/- CD2-myc+ (■, n=18); p53-/- (O, n=11); p53+/- CD2-myc+ (◆, n=34); p53+/- (●, n=24); p53+/+ CD2-myc+ (□, n=10) and p53+/+ (X, n=10) mice. Animals were culled when showing abnormal clinical signs. p53-/- CD2-myc+ animals had a much reduced survival compared to all other littermates.

Of the control groups, 30% of the $p53^{+/+}$/CD2-*myc*$^{+}$ mice died with a latency of between 4 and 8 months and 100% of $p53^{-/-}$ mice died at a latency of 3 to 10 months. These results, shown in Figure 3.1, were comparable with the original reports of these transgenic lines (Stewart *et al.*, 1993; Donehower *et al.*, 1992) and demonstrated the increased kinetics of tumour development in the mice carrying both these genetic lesions. From these observations it was concluded that the presence of the c-*myc* transgene and loss of p53 function acted synergistically during lymphomagenesis.

As evident from Figure 3.1, there was no significant difference in the tumour incidence between CD2-*myc* transgenic animals heterozygous for the p53 null mutation ($p53^{+/-}$ /CD2-*myc*$^{+}$) and CD2-*myc* animals on a wild type p53 background ($p53^{+/+}$/CD2-*myc*$^{+}$) during the 12 month observation period (Mann-Whitney confidence test). The study of these animals was extended further and will be discussed in chapter 5.

3.2.3 Pathology and Histopathology of $p53^{-/-}$/CD2-*myc*$^{+}$ Lymphomas.

CD2-*myc* transgenic mice have been found to develop only thymic lymphoma, consistent with the targeting of the transgene to the T-cell lineage (Stewart *et al.*, 1993). Although lymphoma was the most common neoplasm observed in $p53^{-/-}$ mice, these mice developed a range of tumour types (Donehower *et al.*, 1992). In addition, $p53^{-/-}$ mice frequently developed two or more independent tumour types. Similarly in the current study, 45% of the $p53^{-/-}$ mice developed non-lymphoid tumours, and 27% developed both a lymphoid and co-existing non-lymphoid neoplasm, as shown in Table 3.2. In contrast to $p53^{-/-}$ mice, $p53^{-/-}$/CD2-*myc*$^{+}$ mice developed exclusively thymic lymphoma (Table 3.2). No evidence of a second primary tumour type was observed in any of the $p53^{-/-}$/CD2-*myc*$^{+}$ mice at post-mortem.

The development of lymphoma in the $p53^{-/-}$/CD2-*myc*$^{+}$ mice elicited recognizable clinical signs. Afflicted animals were generally smaller and thinner than littermates with an uneven coat texture and an increased respiratory rate. The movement and responses of ill mice were usually slower, and occasionally animals exhibited a high gait. These clinical signs were similar to those of CD2-*myc* and $p53^{-/-}$ animals which also developed lymphoma.

Table 3.2 Tumour Development in p53$^{-/-}$/CD2-*myc*$^{+}$ Mice and Control Groups.

A. Tumour Types of p53$^{-/-}$/CD2-*myc*$^{+}$ Mice.

Tumour Type	Incidence	Lifespan
Thymic Lymphoma	18/18 (100%)	60 - 86 days

B. Tumour Types of p53$^{-/-}$ Mice.

Tumour Type	Incidence	Lifespan
Thymic lymphoma only	6/11 (55%)	78 - 295 days
Thymic lymphoma & haemangiosarcoma	2/11 (18%)	136 - 201 days
Thymic lymphoma & fibrosarcoma	1/11 (9%)	233 days
Haemangiosarcoma	1/11 (9%)	113 days
Osteosarcoma	1/11 (9%)	237 days

C. Tumour Types of p53$^{+/+}$/CD2-*myc*$^{+}$ Mice.

Tumour Type	Incidence	Lifespan
Thymic lymphoma	3/10 (30%)*	126 - 238 days

*the remaining 70% of p53$^{+/+}$/CD2-*myc*$^{+}$ animals were healthy at the end of the experiment.

Gross pathological examination revealed that the thymus of all p53$^{-/-}$/CD2-*myc*$^{+}$ mice was abnormally enlarged with loss of the normal bilobed structure. In most cases the thymic tumour filled the thoracic cavity. Histopathological examination of the thymic tumours revealed that the normal architecture of the thymus tissue was replaced by large pleomorphic lymphoblasts showing an extremely high mitotic rate. A 'starry sky' pattern due to marked cell death and increased macrophage activity was observed. The histopathological findings were consistent with a diagnosis of lymphoblastic thymic lymphoma and were similar to those previously found in CD2-*myc* tumours (Stewart *et al.*, 1993).

3.2.4 Metastatic Spread In p53$^{-/-}$/CD2-*myc*$^{+}$ Lymphomas.

A characteristic feature of the lymphomas which developed in the p53$^{-/-}$/CD2-*myc*$^{+}$ mice was the high degree of metastasis. Gross pathology showed that, without exception, all p53$^{-/-}$/CD2-*myc*$^{+}$ thymic tumours were accompanied by metastatic spread to the secondary lymphoid tissues. The spleen, mesenteric and subcutaneous lymph nodes were always enlarged, generally grossly so. In addition, involvement of the liver and kidneys was often observed, with an increase in organ size and associated pallor. Histopathological diagnosis of these tissues confirmed that the lymphoblastic lymphomas were associated with multiorgan involvement. The spleen was usually replaced by lymphoblasts and often had areas of extra-medullary haemopoiesis. The normal architecture of the lymph nodes was altered by the presence of large lymphoblasts and the liver and kidneys showed varying degrees of lymphoblastic infiltration. Diffuse infiltration of the bone marrow was frequently observed.

In contrast to the p53$^{-/-}$/CD2-*myc*$^{+}$ mice, gross pathological examination of parental p53$^{+/+}$/CD2-*myc*$^{+}$ and p53$^{-/-}$ groups did not always demonstrate metastatic spread and when metastasis did occur it was seldom as advanced as that seen in the p53$^{-/-}$/CD2-*myc*$^{+}$ group. Four out of nine lymphomas which developed in the p53$^{-/-}$ controls were restricted to the thymus with little or no involvement of other tissues. The other lymphomas in this group exhibited obvious splenic involvement but, in general, had limited spread to lymph nodes and non-lymphoid tissues. Two out of three of the p53$^{+/+}$/CD2-*myc*$^{+}$ lymphomas did demonstrate gross enlargement of the spleen and lymph nodes but with little involvement of the thymus. In these instances the architecture of the thymus was still recognizable although histopathological examination did denote tumour activity.

3.2.5 CD2-*myc* Transgene Expression.

Previous studies have shown that although high levels of human c-*myc* were observed in thymic tumours of CD2-*myc* transgenic mice, transgene expression was not detected in preneoplastic tissues (Stewart *et al.*, 1993). Therefore to confirm that the CD2-*myc*

transgene was transcriptionally active in the p53$^{-/-}$/CD2-*myc*$^{+}$ tumours, Northern analysis was carried out on these tissues. As Figure 3.2 illustrates, all p53$^{-/-}$/CD2-*myc*$^{+}$ thymic lymphomas examined (14/14), expressed detectable levels of the CD2-*myc* transcript. Transgene expression was also detected in the kidney tissue of some of the p53$^{-/-}$/CD2-*myc*$^{+}$ mice (Mrs. M. O'Hara, personal communication; Blyth *et al.*, 1995). This result correlates well with the metastatic spread to kidney tissue in these mice. It can be concluded from these results that overexpression of the CD2-*myc* transgene was contributing to the development of lymphoid tumours in p53$^{-/-}$/CD2-*myc*$^{+}$ mice.

3.2.6 Immunophenotype Analysis.

Cells prepared from thymus or lymph node tumour tissue were analysed by flow cytometry to ascertain the cell lineage of the lymphomas. The panel of antibodies used in this analysis is given in Table 2.1. Isotype matched antibody controls were used for each flourochrome to exclude non-specific staining. All p53$^{-/-}$/CD2-*myc*$^{+}$ lymphomas studied were found to be of T-cell origin, staining positive for the CD3 T-cell specific marker (Figure 3.3.a). Furthermore, none of the lymphomas stained positive for the B-cell marker CD45R (B220). The T-cell composition of the tumours was confirmed by TcR rearrangement analysis (Mrs. A. Terry, personal communication; Blyth *et al.*, 1995).

The tumours were also stained for expression of the T-cell surface antigens CD4 and CD8 (Figure 3.3.b). The most common phenotype detected in p53$^{-/-}$/CD2-*myc*$^{+}$ tumours was a CD4^{+}CD8^{+} double-positive phenotype, with 9 out of 17 lymphomas consisting predominantly of CD4^{+}CD8^{+} tumour cells (Table 3.3). None of the p53$^{-/-}$/CD2-*myc*$^{+}$ tumours were of an exclusive single-positive mature phenotype, either CD4^{-}CD8^{+} or CD4^{+}CD8^{-}. One tumour was double negative for CD4 and CD8 co-receptor expression.

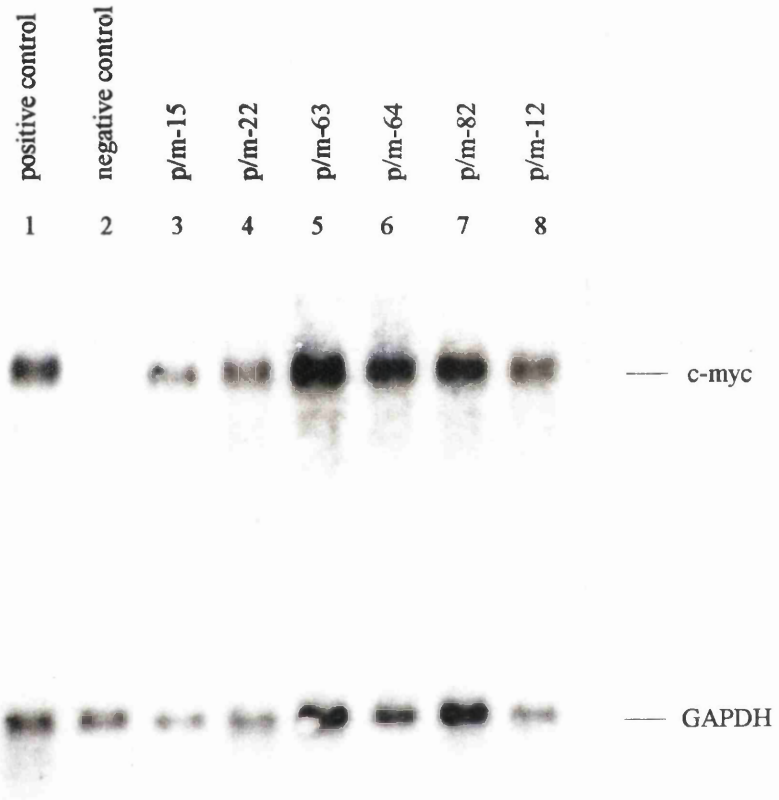

Figure 3.2. Northern Blot Analysis of p53-/- CD2-myc+ Thymic Tumours.

The thymic tumours of p53-/- CD2-myc+ mice were assessed for CD2-myc transgene expression using a probe to human c-myc exon 3. Lanes 1 and 2 are positive and negative controls respectively. Lanes 3 to 8 represent a selection of p53-/- CD2-myc+ tumours, the code name for each tumour is given. Samples were also probed for the murine GAPDH gene to confirm the presence of RNA.

Similar to that observed with the p53$^{-/-}$/CD2-*myc*$^{+}$ tumours, the predominant phenotype of both CD2-*myc* and p53$^{-/-}$ tumours was CD4^{+}CD8^{+} (Table 3.3). Due to the low tumour number in the p53$^{+/+}$/CD2-*myc*$^{+}$ control group of the current study, CD2-*myc* tumour phenotype data has been taken from a separate study (Cameron *et al.*, 1996) for the purpose of comparison. More than 20% (18/89) of CD2-*myc* tumours demonstrated a CD4^{-}CD8^{+} single-positive phenotype. The absence of mature single-positive phenotypes in p53$^{-/-}$/CD2-*myc*$^{+}$ tumours therefore is in contrast to that seen in CD2-*myc* tumours.

Table 3.3 The CD4/CD8 Phenotype of p53$^{-/-}$/CD2-*myc*$^{+}$ Lymphoid Tumours as Analysed by Flow Cytometry.

Tumour phenotype	CD4^{+}CD8^{+}	CD4^{+}CD8^{-}	CD4^{-}CD8^{+}	CD4^{-}CD8^{-}	mixed[a]
No. (%) of p53$^{-/-}$/CD2-*myc*$^{+}$ lymphomas exhibiting phenotype	9/17 (53%)	0	0	1/17 (6%)	7/17 (41%)
No. (%) of p53-/- lymphomas exhibiting phenotype [b]	8/16 (50%)	0	1/16 (6%)	2/16 (12%)	5/16 (31%)
No. (%) of CD2-*myc* lymphomas exhibiting phenotype [c]	61/89 (69%)	1/89 (1%)	18/89 (20%)	1/89 (1%)	8/89 (9%)

[a] mixed tumour phenotype refers to instances where more than one major population exists.

[b] these results are a combination of the current cohort of p53$^{-/-}$ lymphomas (n=8) and of other previously unpublished results taken from a control cohort (n=8).

[c] results from Cameron *et al.*, 1996 (see text).

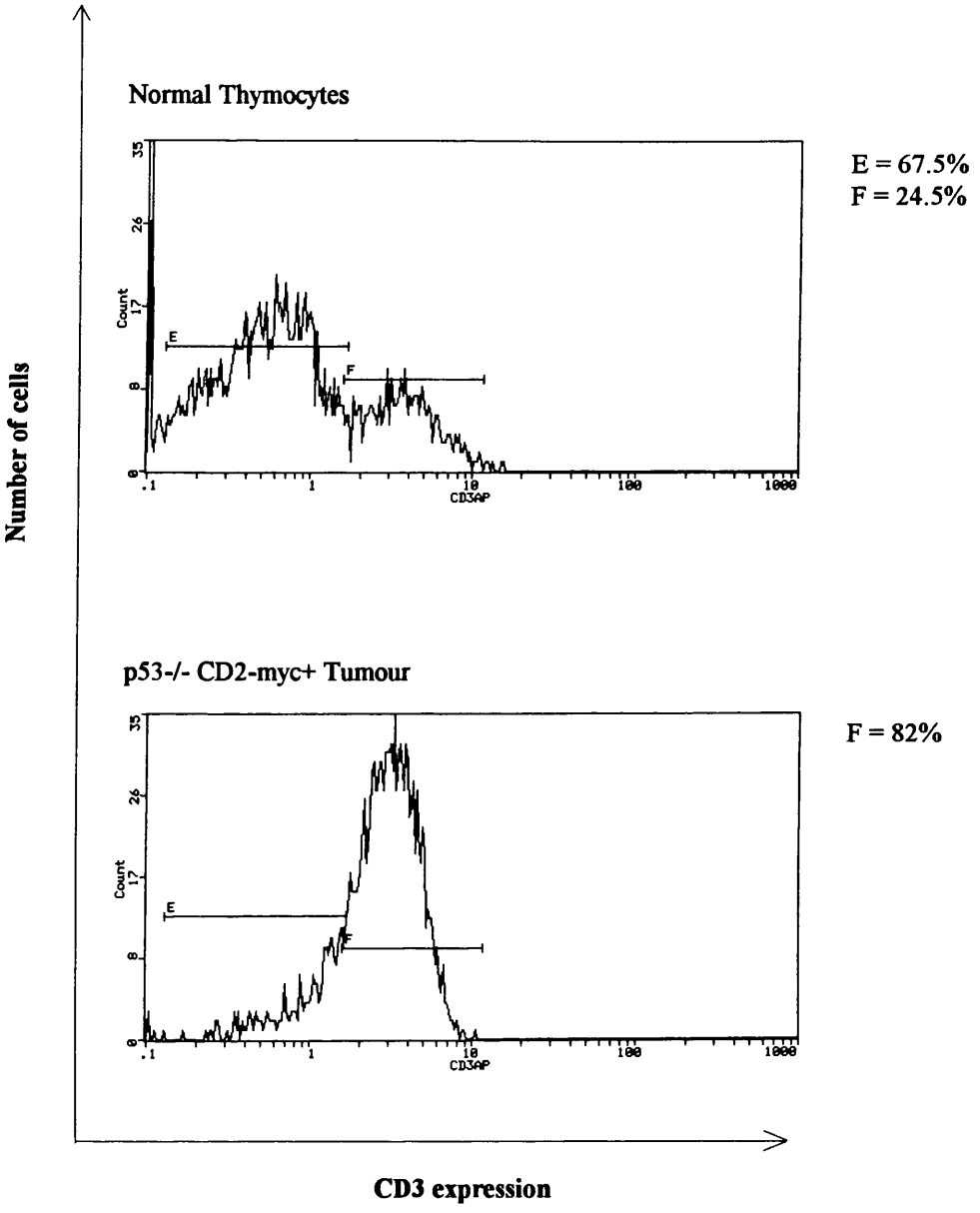

Figure 3.3.a. Flow Cytometry Analysis of p53-/- CD2-myc+ Lymphomas.

This figure illustrates the CD3 surface marker expression of normal mouse thymocytes (top) and a representative p53-/- CD2-myc+ lymphoma (bottom). Normal thymocytes had both a low and high peak of CD3 surface expression. All p53-/- CD2-myc+ thymic lymphomas were positive for CD3 surface expression and displayed a single peak of CD3 expression. The percentage of positive cells low for CD3 expression (E) and high for CD3 expression (F) is shown.

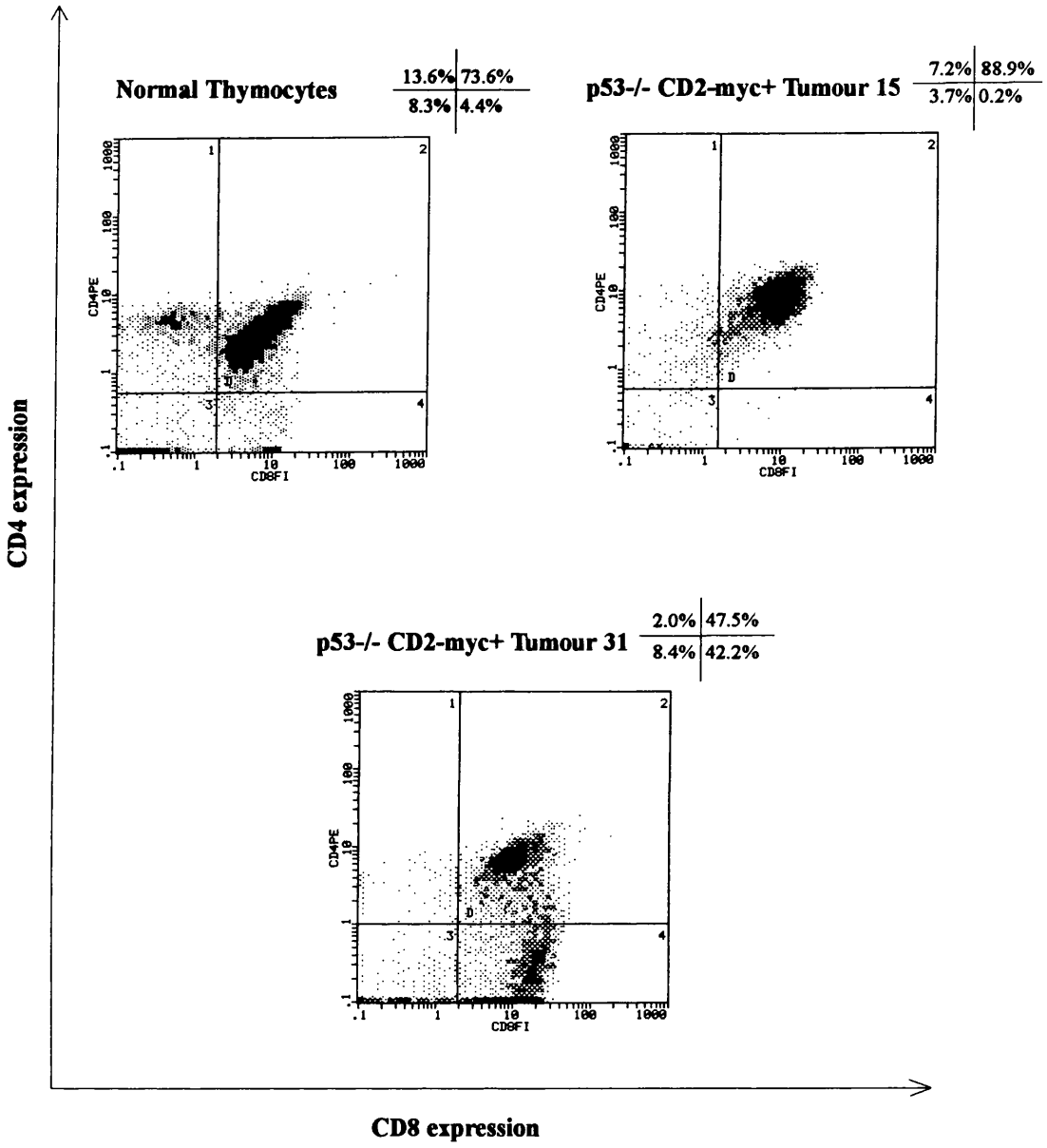

Figure 3.3.b. Flow Cytometry Analysis of p53-/- CD2-myc+ Lymphomas.

This figure illustrates the CD4 and CD8 surface marker expression of normal mouse thymocytes (top left) and two p53-/- CD2-myc+ lymphomas. The majority of p53-/- CD2-myc+ thymic tumours were CD4+CD8+ tumours (top right) although a number of tumours consisted of a mixed population phenotype (bottom). The percentage of positive cells in each quadrant is shown.

3.2.7 Tumour Clonality.

A prominent feature of the $p53^{-/-}$/CD2-*myc*$^{+}$ lymphomas was the number of tumours (7/17) with more than one discrete T-cell population (Table 3.3). These tumours frequently (4/7) consisted of a $CD4^{+}CD8^{+}$ population and a $CD4^{-}CD8^{+}$ population (Figure 3.3.b). Mixed T-cell populations were also observed in $p53^{-/-}$ lymphomas (5/16). Although mixed T-cell populations were not unique to the $p53^{-/-}$/CD2-*myc*$^{+}$ cohort, the frequency with which they occurred may reflect an increase in the clonal complexity of the tumours. Clonal complexity was further characterised by investigating IgH rearrangements by Southern blot analysis (carried out by Mrs. A. Terry). Rearrangement of the IgH gene was a frequent event in the lymphomas which were studied. The oligoclonal pattern of these tumours confirmed that the $p53^{-/-}$/CD2-*myc*$^{+}$ tumours were generally of greater clonal complexity than the parental $p53^{+/+}$/CD2-*myc*$^{+}$ and $p53^{-/-}$ cohorts (Figure 3.4).

3.2.8 Cell Line Establishment.

Lymphoid tumour cells were routinely isolated from thymic lymphomas by disaggregation and separation on a ficoll density gradient. The *in vitro* growth potential of these cells was investigated in long term cell line culture. All of the $p53^{-/-}$/CD2-*myc*$^{+}$ tumours which were tested (13/13), readily established in long term culture without addition of growth factor. This result was in marked contrast to tumour cells isolated from CD2-*myc* mice which always failed to establish in culture under these conditions (6/6). Long term cell line establishment of $p53^{-/-}$ lymphoma cells was frequently achieved. Three out of 6 $p53^{-/-}$ lymphomas from the current study group and 6/7 from previous experiments successfully established *in vitro*. Of the $p53^{-/-}$ lymphomas which failed to establish in culture, 3/4 were isolated from mice which presented with a co-existing haemangiosarcoma at post-mortem. The establishment of $p53^{-/-}$ tumour cells was also found to correlate with the age at which these mice developed lymphomas. Lymphomas developing in younger mice were found to establish more frequently than lymphoma cells isolated from older animals.

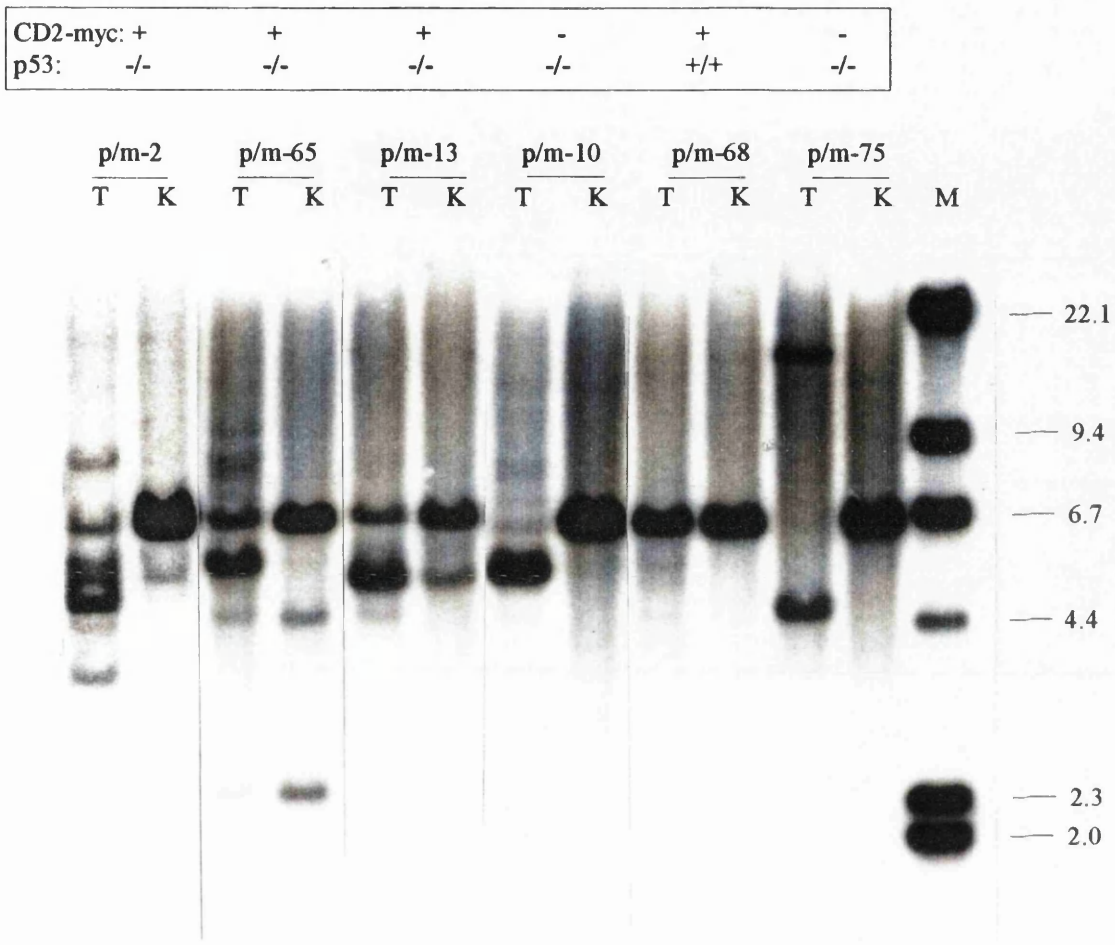

Figure 3.4. Increased Clonal Complexity of p53-/- CD2-myc+ Tumours.

IgH rearrangements were used to assess the clonality of thymic tumours (T) and kidney (K) tissues of p53-/- CD2-myc+ mice and control littermates. Three representative p53-/- CD2-myc+ tumours and three control genotypes are shown in this figure, adapted from Blyth *et al.*, 1995. The tumour name and mouse genotype for each sample is given above the blot. It can be seen that p53-/- CD2-myc+ tumours were generally of greater clonal complexity than tumours from parental controls. These Southern blots were carried out by Mrs. A. Terry and have been used here with Anne's kind permission.

3.2.9 Immunophenotype Analysis of $p53^{-/-}$/CD2-*myc*$^{+}$ Cell Lines.

Immunophenotype analysis was carried out on a proportion of the $p53^{-/-}$/CD2-*myc*$^{+}$ cell lines. Six lines which had been established for a period of greater than 6 months were chosen as it was thought that these lines would be stable with respect to T-cell differentiation. Particular attention was given to those cell lines which had been derived from lymphomas exhibiting mixed T-cell populations. All of the $p53^{-/-}$/CD2-*myc*$^{+}$ cell lines analysed contained monoclonal populations with respect to CD4 and CD8 phenotype, results are shown in Table 3.4.

Table 3.4 Immunophenotype of Established Tumour Cell Lines.

Mouse Identification Number	Mouse Genotype	Tumour Populations[#]	Cell Line Populations
p/m-31	$p53^{-/-}$/CD2-*myc*$^{+}$	$CD4^{+}CD8^{+}$* & $CD4^{-}CD8^{+}$	$CD4^{+}CD8^{+}$
p/m-63	$p53^{-/-}$/CD2-*myc*$^{+}$	$CD4^{+}CD8^{+}$* & $CD4^{-}CD8^{+}$	$CD4^{+}CD8^{+}$
p/m-64	$p53^{-/-}$/CD2-*myc*$^{+}$	$CD4^{-}CD8^{-}$* & $CD4^{-}CD8^{+}$	$CD4^{-}CD8^{-}$
p/m-65	$p53^{-/-}$/CD2-*myc*$^{+}$	$CD4^{+}CD8^{+}$	$CD4^{+}CD8^{-}$
p/m-85	$p53^{-/-}$/CD2-*myc*$^{+}$	$CD4^{+}CD8^{+}$* & $CD4^{-}CD8^{-}$	$CD4^{+}CD8^{+}$
p/m-103	$p53^{-/-}$/CD2-*myc*$^{+}$	$CD4^{-}CD8^{-}$	$CD4^{-}CD8^{-}$
p/m-40	$p53^{-/-}$	$CD4^{+}CD8^{+}$* & $CD4^{-}CD8^{+}$	$CD4^{+}CD8^{+}$ & $CD4^{-}CD8^{+}$
p/m-97	$p53^{-/-}$	$CD4^{+}CD8^{+}$ & $CD4^{-}CD8^{+}$*	$CD4^{-}CD8^{lo}$ & $CD4^{-}CD8^{+}$

[#] represents only principal clonal populations of greater than 30%

* indicates the major tumour population

In five of the six lines, the cell line population was a reflection of the main tumour population (Table 3.4). In one cell line, p/m-65, a mature $CD4^{+}CD8^{-}$ population was observed which was in contrast to the immature $CD4^{+}CD8^{+}$ population of the tumour. This may have been due to downregulation of CD8 surface expression *in vitro*, resulting

in the differentiation of the cells during culture. Alternatively, a minor population (15%) of $CD4^+CD8^-$ cells which was present in the tumour may have outgrown the main $CD4^+CD8^+$ tumour population *in vitro*. Two of the $p53^{-/-}$ cultured cell lines were also immunophenotyped (Table 3.4). Unlike the $p53^{-/-}$/CD2-*myc*$^+$ cell lines, the $p53^{-/-}$ lines were not monoclonal. One cell line had a diffuse $CD4^+CD8^+$ population and a separate $CD4^-CD8^+$ population. Both of these populations were present in the original tumour.

3.2.10 *In situ* Apoptosis Studies.

In an attempt to investigate the apoptotic status of the $p53^{-/-}$/CD2-*myc*$^+$ lymphomas, tissue sections were examined by the TUNEL technique. This technique involves the incorporation of labelled nucleotide into the DNA strand breaks of apoptotic cells using terminal deoxynucleotidyl transferase. The labelled nucleotide can then be identified by conventional immunocytochemistry (Gavrieli *et al.*, 1992). Tissue sections of $p53^{-/-}$ /CD2-*myc*$^+$ lymphomas were stained by this technique and compared against $p53^{-/-}$ and CD2-*myc* lymphomas (Figure 3.5). Normal mouse thymus was used as a control. Apoptosis was found to occur in the lymphomas of all three tumour groups tested. Two staining patterns were observed, staining of single cells and staining of apoptotic bodies engulfed within phagocytic cells. A high degree of heterogeneity was found in the thymic tumours of all genotypes. Some tumours were found to contain many positive cells and/or apoptotic bodies while some tumours had very few positively stained cells (Figure 3.5). Detailed examination of the tissue sections and quantification of the apoptotic indices revealed that there was no obvious difference in the staining patterns or number of apoptotic cells of $p53^{-/-}$/CD2-*myc*$^+$ tumours versus CD2-*myc* ($p53^{+/+}$) and $p53^{-/-}$ lymphomas (Figure 3.6).

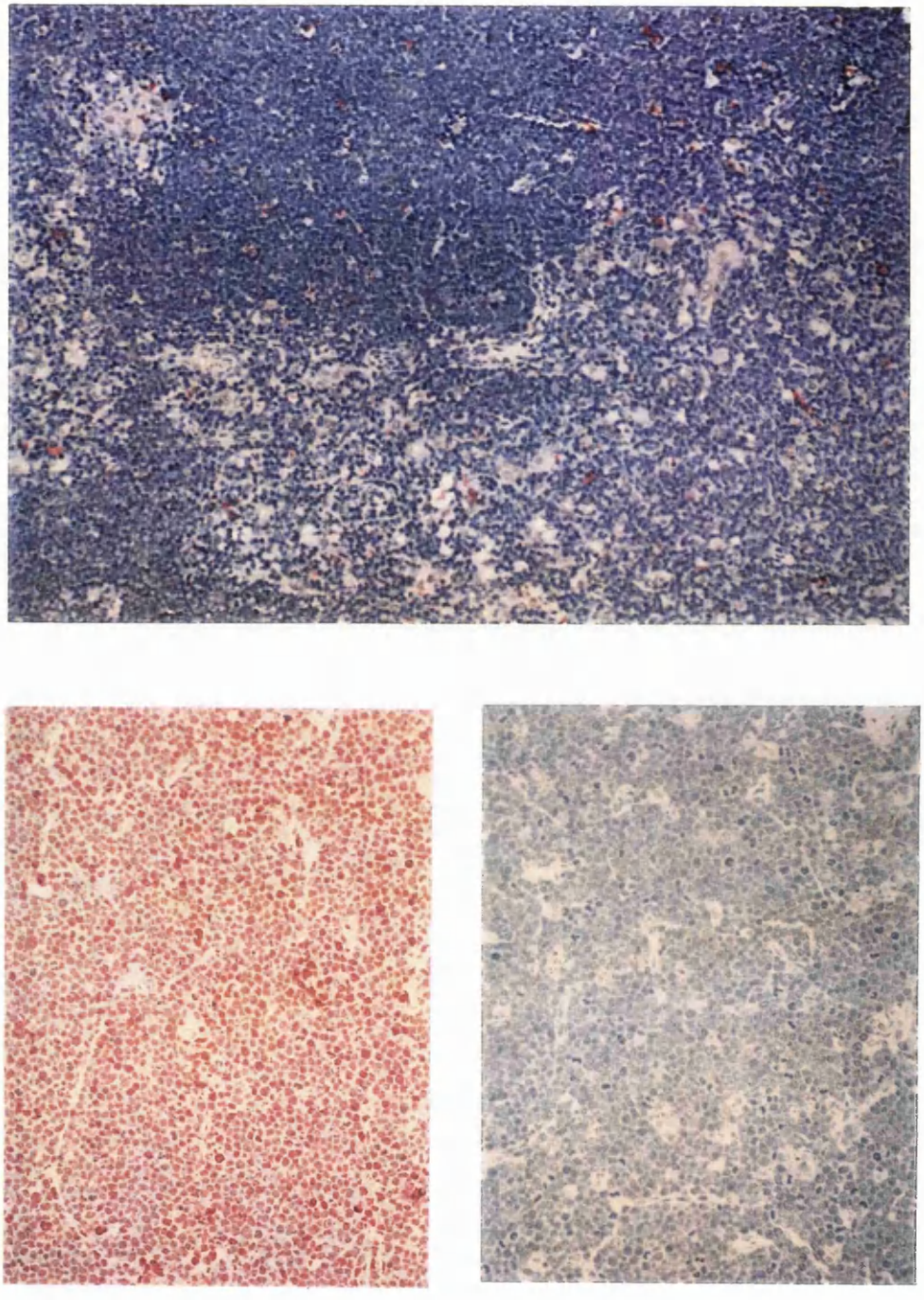

Figure 3.5.a. TUNEL Analysis, Control Sections.

Top: Normal mouse thymus (x50); Bottom left: Positive control, tumour section treated with DNase (x50). Bottom right: Negative control, tumour section to which TdT/dUTP enzyme mix was not added (x50). (Original in colour).

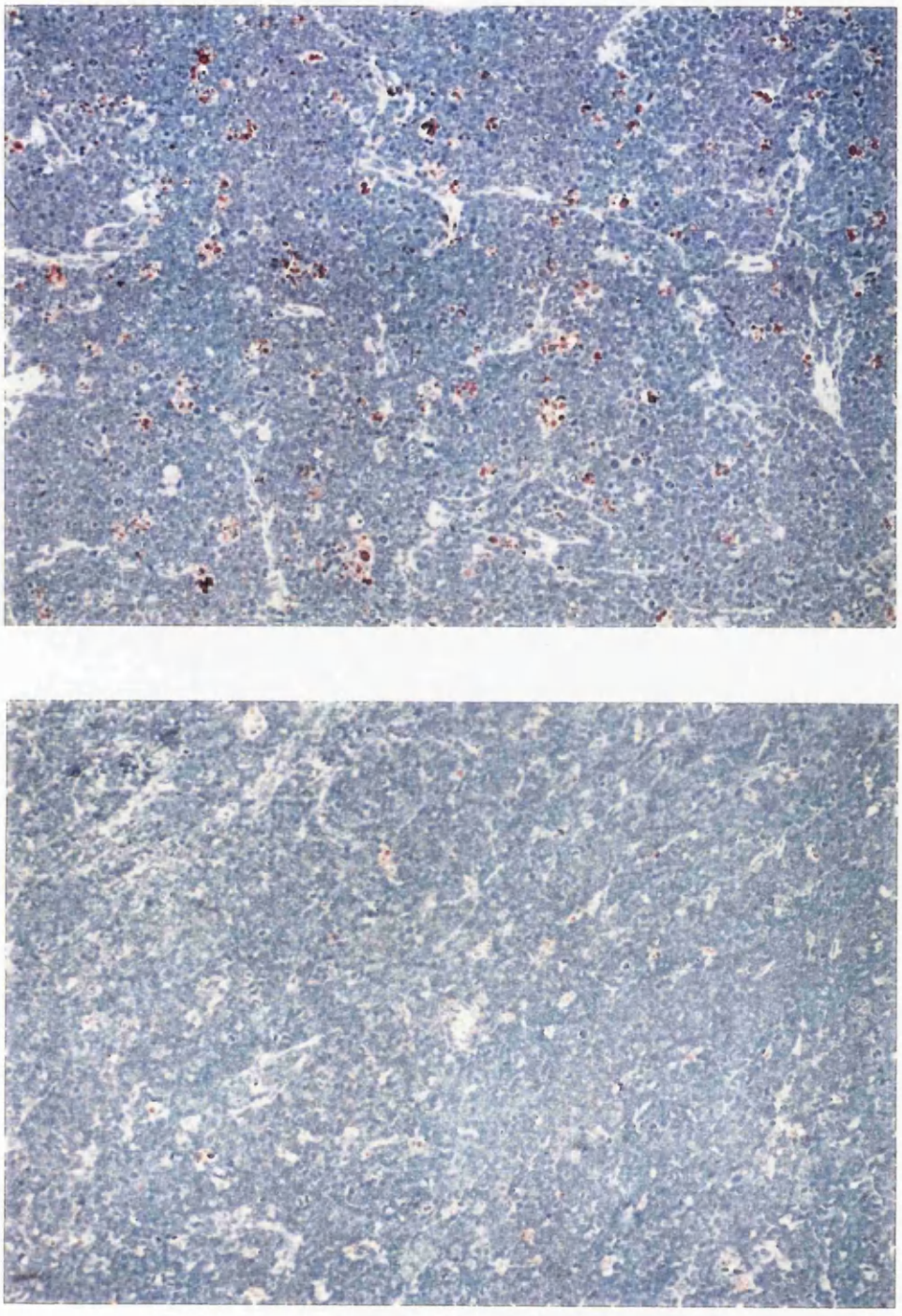

Figure 3.5.b. TUNEL Analysis of p53-/- CD2-myc+ Tumour Sections.

This figure illustrates TUNEL-stained sections of two p53-/- CD2-myc+ thymic tumours (x50). Apoptotic cells appear as red stained cells. The level of apoptosis was heterogenous within the p53-/- CD2-myc+ tumour cohort as shown by these two sections. (Original in colour).

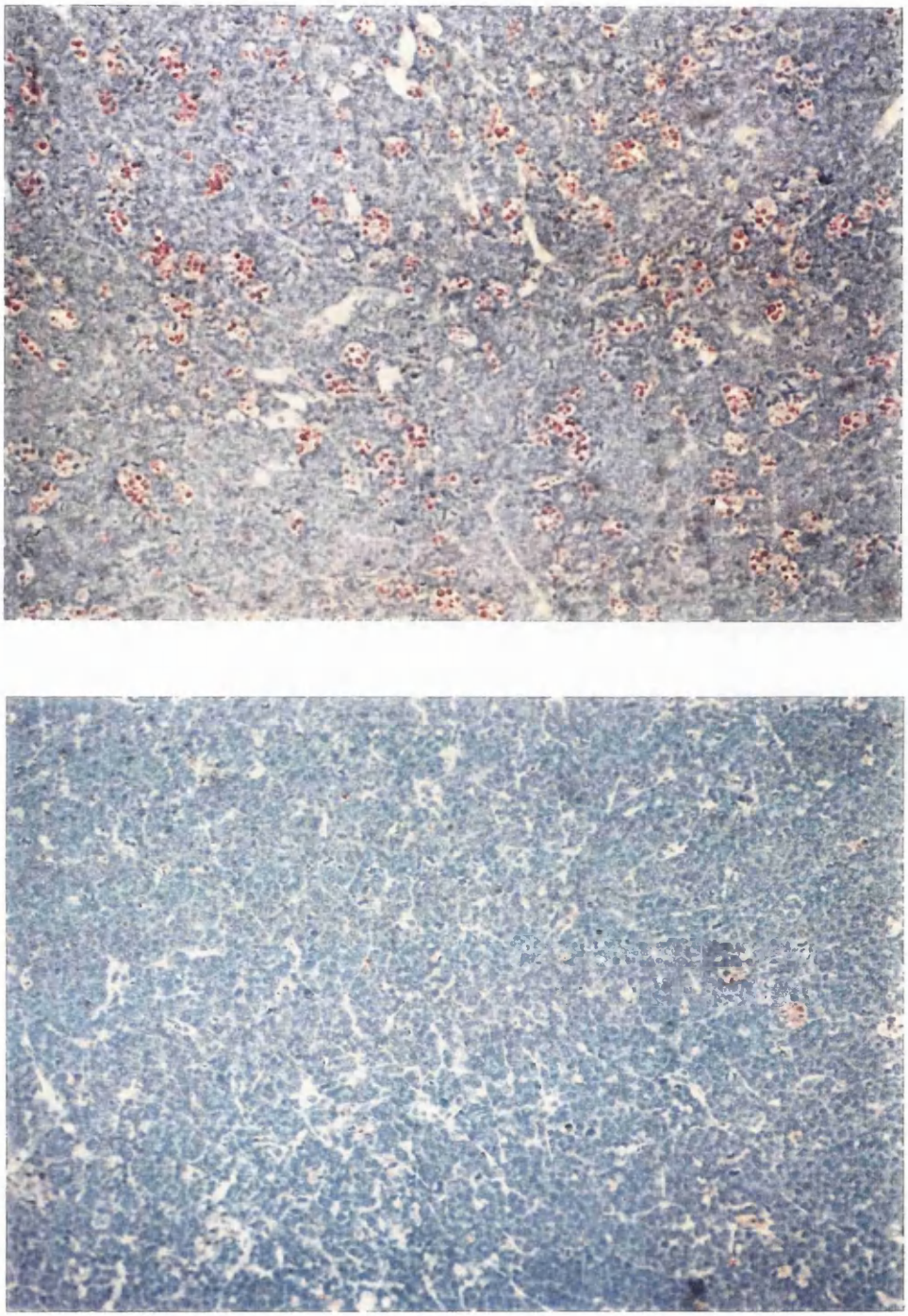

Figure 3.5.c. TUNEL Analysis of p53-/- Tumour Sections.

TUNEL-stained sections of two p53-/- thymic tumours displaying the heterogeneity of staining patterns observed in this tumour cohort (x50, Original in colour).

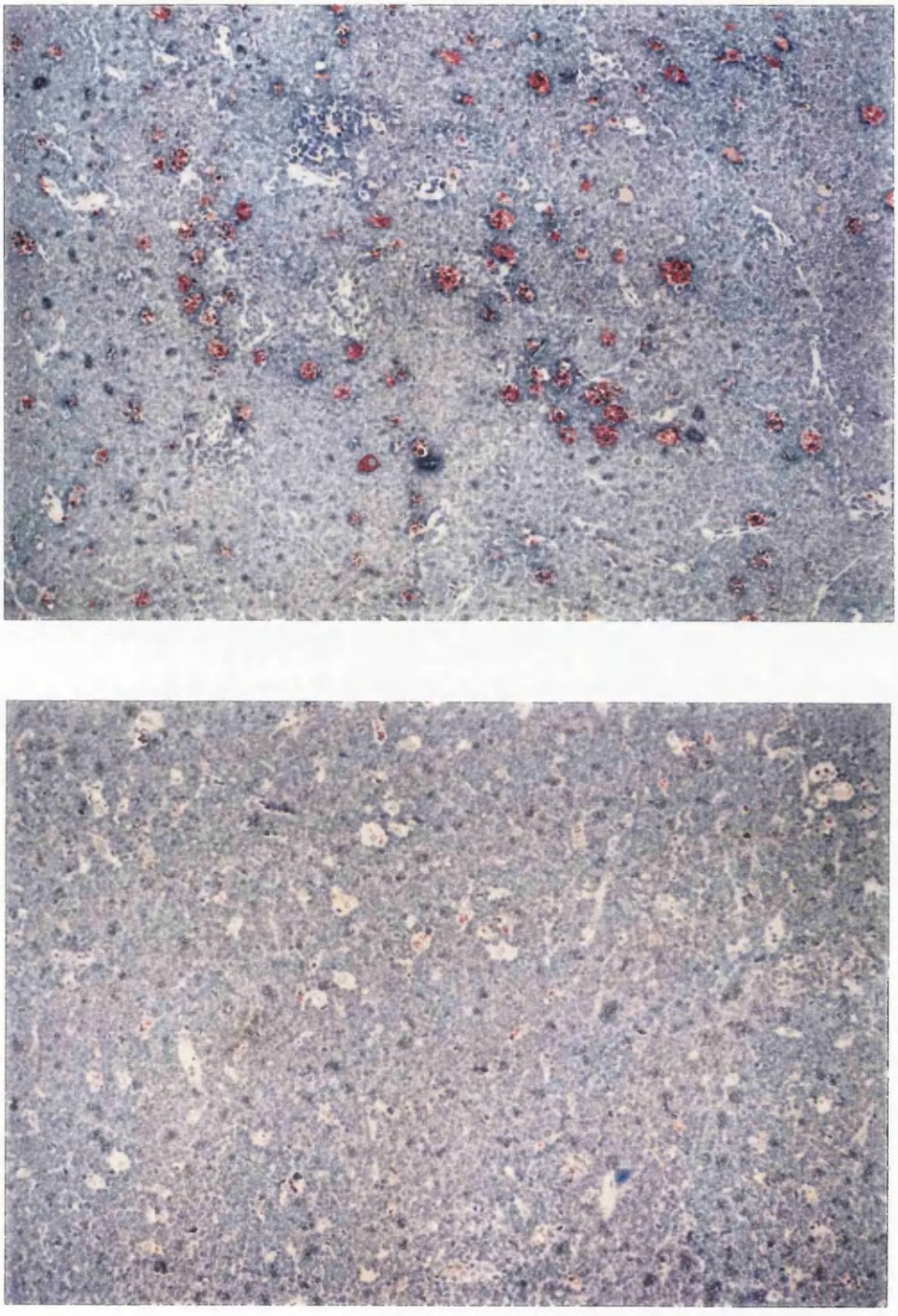

Figure 3.5.d. TUNEL Analysis of CD2-myc Tumour Sections.

TUNEL-stained sections of two CD2-myc thymic tumours (x50). The level of apoptosis was heterogenous within this tumour group. Obvious macrophage engulfment of apoptotic bodies can be observed in the top photograph. (Original in colour).

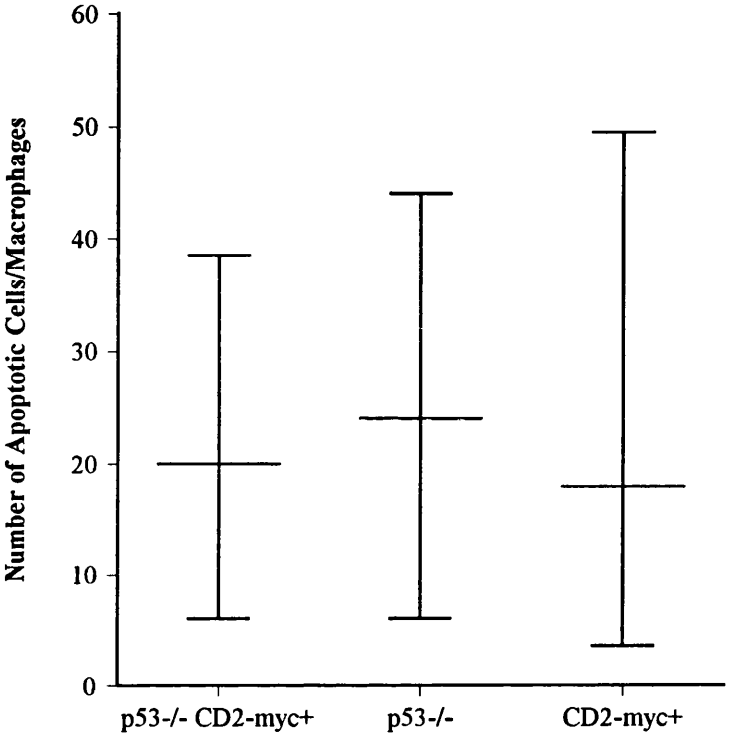

Figure 3.6. Assessment of Apoptosis in p53-/- CD2-myc+ Thymic Tumours.

The level of apoptosis in each of fourteen p53-/- CD2-myc+ tumours, six p53-/- tumours and seven CD2-myc+ tumours was assessed using the TUNEL method. Positive stained cells were counted in six high power light microscope fields (x400) for each tumour section and an average taken. Each cell or macrophage containing apoptotic bodies was regarded as one positive count. The standard error bars show the range of the total number of apoptotic cells which were seen for each genotype. There was no statistical difference in the level of positive apoptotic cells in the p53-/- CD2-myc+ tumour group versus the control groups (Mann-Whitney confidence test).

3.3 DISCUSSION.

3.3.1 Synergy Between CD2-*myc* and p53 Loss in T-Lymphomagenesis.

The combined action of c-*myc* overexpression and lack of the p53 tumour suppressor gene product was investigated in an *in vivo* tumourigenesis model. Transgenic mice positive for both a CD2-*myc* transgene and homozygous p53 null mutation were generated by crossing these parental strains together (Stewart *et al.*, 1993; Donehower *et al.*, 1992). The co-existence of these genetic lesions predisposed the 'double transgenic' animals to rapid lymphoma development. Relative to parental strains, the $p53^{-/-}$/CD2-myc^{+} mice developed lymphomas with increased frequency at a significantly accelerated rate. All $p53^{-/-}$/CD2-myc^{+} mice were found to succumb to T-cell lymphomagenesis before 13 weeks of age. It was concluded from these observations that overexpression of c-*myc* and loss of p53 function could act synergistically to promote T-lymphoid tumours in mice.

Collaboration of c-*myc* and p53 mutations in tumourigenesis was previously suggested from the observed co-existence of these mutations in a number of human cancers, particularly lymphomas (Farrell *et al.*, 1991; Gaidano *et al.*, 1991; Farrugia *et al.*, 1994; Chang *et al.*, 1994; Ballerini *et al.*, 1992). Moreover, p53 mutations were found to occur in B-cell tumours arising in Eμ-*myc* transgenic mice (Scheuermann & Bayer, 1992; Adams & Cory, 1992). In contrast however, p53 mutations were an infrequent event in c-*myc* induced mouse plasmacytomas (Gutierrez *et al.*, 1992). Direct confirmation that a co-operative relationship does exist between these genetic mutations has been provided by the current $p53^{-/-}$/CD2-myc^{+} model.

3.3.2 $p53^{-/-}$/CD2-myc^{+} Mice Develop Malignant Thymic Lymphomas.

Consistent with the observation that p53 mutations are common in a wide spectrum of human cancers (Hollstein *et al.*, 1991; Jego *et al.*, 1993), p53 null mice develop a range of tumour types (Donehower *et al.*, 1992; Purdie *et al.*, 1994; Jacks *et al.*, 1994). In contrast, $p53^{-/-}$/CD2-myc^{+} mice developed only one tumour type, namely lymphoblastic

thymic lymphoma. The CD2-*myc* transgene is targeted to the T-cell lineage and as such CD2-*myc* transgenic mice develop only T-cell lymphomas (Stewart *et al.*, 1993). Therefore the presence of the CD2-*myc* transgene has restricted the tumour phenotype of the 'double transgenic' mice. It is possible however that the early age at which the $p53^{-/-}$/CD2-myc^{+} mice died may have circumvented the latency time necessary for other neoplasms to manifest. In general, sarcomas and carcinomas do not develop in $p53^{-/-}$ mice until after a latency of 16 weeks.

The $p53^{-/-}$/CD2-myc^{+} lymphomas were observed to be highly metastatic exhibiting multiorgan involvement and associated lymphoblastic leukaemia. This high level of metastatic spread was not always displayed by the control $p53^{+/+}$/CD2-myc^{+} and $p53^{-/-}$ mice. Thus the combination of a p53 null background and overexpressed c-*myc* produced a highly malignant tumour phenotype. Based on flow cytometry analysis and immunoglobulin gene rearrangements, the $p53^{-/-}$/CD2-myc^{+} lymphomas were generally of higher clonal complexity than the control littermates. The oligoclonality of these tumours however indicated that these two events alone were insufficient for complete neoplastic transformation. This interpretation was confirmed by infection of $p53^{-/-}$/CD2-myc^{+} mice with MoMuLV which resulted in a further acceleration of tumour onset (see chapter 6).

3.3.3 Synergy Occurs Between the p53 null Mutation and Several c-*myc* Transgenes.

Subsequent to this study, two independent laboratories reported similar findings. By crossing p53 null mice with Eμ-*myc* transgenic mice, Hsu and co-workers demonstrated that these lesions also act cooperatively to reduce the latency time of B-cell lymphomagenesis. The tumours arising in the $p53^{+/-}$/Eμ-*myc* mice were high grade diffuse large cell malignant lymphomas similar to Eμ-*myc* mice (Hsu *et al.*, 1995). The effects of the MMTV/c-*myc* transgene on a p53 null background was investigated in a separate study by Elson *et al.* (1995). This transgene was expressed in a variety of tissues and the authors postulated that the co-existing effects of these lesions could be assessed in a number of cell types. Most of the $p53^{+/-}$/MMTV/c-*myc* transgenic animals

however developed pre-T and T-cell lymphomas even although the predominant tumour type of MMTV/c-*myc* mice was mammary carcinoma (Stewart *et al.*, 1984a). The lack of acceleration in mammary tumour formation in these mice may be a reflection that mouse mammary tissue is not particularly susceptible to the effects of p53 loss, consistent with the rare incidence of mammary tumours in p53 null animals (Donehower *et al.*, 1992; Purdie *et al.*, 1994). However it was suggested by Elson and co-workers that this result may be due to a cell type specific collaboration between the c-*myc* and p53 null lesions (Elson *et al.*, 1995).

In contrast to $p53^{-/-}$/CD2-myc^{+} mice, no co-operation was observed between the CD2-*myc* transgene and the heterozygote p53 mutation during the twelve month observation period of this study (Figure 3.1). By comparison, synergy with each of the MMTV/c-*myc* and Eμ-*myc* transgenes was demonstrated in animals which were genotypically heterozygous for the p53 null mutation (Elson *et al.*, 1995; Hsu *et al.*, 1995). As all three studies utilized the same strain of p53 null mouse (Donehower *et al.*, 1992), it seems likely that these contrasting results reflect differences in either the c-*myc* transgenes and/or the targeted tissue type. The lack of acceleration in $p53^{+/-}$/CD2-myc^{+} mice compared with $p53^{-/-}$/CD2-myc^{+} mice suggests that loss of the wild type p53 allele may be an important event for the initiation of these tumours. Similarly, loss of wild type p53 was observed during the tumourigenesis process in $p53^{+/-}$/MMTV/c-*myc* and $p53^{+/-}$/Eμ-*myc* mice resulting in a reduction to homozygosity in a significant proportion of the heterozygote tumours (Elson *et al.*, 1995; Hsu *et al.*, 1995).

3.3.4 Mechanisms of Synergy in $p53^{-/-}$/CD2-myc^{+} Accelerated Lymphomas.

Several plausible, but not exclusive, hypotheses could account for the genetic synergy between the CD2-*myc* and $p53^{-/-}$ mutations. Firstly, p53 and c-*myc* proteins have been shown to transcriptionally modulate the activity of each other. *In vitro* assays demonstrated that wild type p53, but not mutant p53, specifically repressed transcription from the c-*myc* promoter (Moberg *et al.*, 1992; Ragimov *et al.*, 1993). These results explain the observation that induced wild type p53 activity downregulated c-*myc* expression in M1 myeloid leukaemia cells during p53 mediated growth arrest (Levy *et*

al., 1993). Recent *in vitro* studies showed that high levels of c-*myc* could abrogate p53-induced growth arrest which may explain why repression of c-*myc* is important during p53-mediated growth arrest (Hermeking *et al.*, 1995). The c-*myc* protein in turn, has been shown to transactivate both the murine and human p53 gene by directly binding to a recognition motif in the p53 promoter (Reisman *et al.*, 1993; Roy *et al.*, 1994). The paradoxical roles for c-*myc* and p53 at the transcriptional level suggests a negative feedback loop whereby inappropriate expression of c-*myc* would be regulated by p53 under normal circumstances. Loss of p53 function may result therefore in uncontrollable c-*myc* expression leading to a highly proliferative state. As the CD2-*myc* transgene retains the P1 regulatory sequences it is possible that p53 can exert a degree of control on transgene expression.

Wild type p53 functions as a G1 cell cycle checkpoint, monitoring the integrity of the genome (Lane, 1992) and mediating growth arrest on cellular insult (Kastan *et al.*, 1991a). It has been suggested that loss of p53 function creates a situation where genetic instability occurs allowing increased accumulation of oncogenic mutations in the cell (Lane, 1992). With regard to the $p53^{-/-}$/CD2-myc^{+} transgenic model, this hypothesis could imply that loss of p53 acts by promoting the survival of cells containing mutations in other *myc*-collaborating genes. It is noteworthy to recall that the CD2-*myc* transgene is not constitutively active in transgenic tissues (Stewart *et al.*, 1993). It is possible that conditional transgene expression may rely on the stochastic activation of the transgene by unknown genetic events which need not be oncogenic but would be related to chromatin configuration at the transgenic loci. Genetic instability created by p53 loss in $p53^{-/-}$/CD2-myc^{+} cells could permit survival of cells with sustained chromosomal damage which may lead to the activation of the CD2-*myc* transgene. In this way, p53 loss would not have direct significance with respect to tumourigenesis. Recent reports demonstrated that loss of p53 function in null mice was not associated with an increased accumulation of mutations (Sands *et al.*, 1995; Nishino *et al.*, 1995). These results would support the proposal that the p53 null mutation acts to directly synergize with the CD2-*myc* transgene rather than merely allowing other collaborating or activating mutations to occur. It should be noted nevertheless that the studies by Sands *et al.* and Nishino *et al.*

did not account for all possible chromosomal aberrations and that undetected mutational events may have occurred.

A report by Metz and colleagues demonstrated that p53 loss can directly contribute to immortalisation and tumourigenesis, possibly by abrogating an intrinsic senescence pathway (Metz *et al.*, 1995). Studies of mouse embryo fibroblasts isolated from p53 null mice demonstrating increased *in vitro* growth without crisis (Harvey *et al.*, 1993c; Tsukada *et al.*, 1993) support the idea of wild type p53 as an enforcer of senescence. Metz *et al.* found that although p53 loss greatly facilitated immortalisation and transformation of haematopoietic cells, it was in itself insufficient and required expression of both *myc* and *raf* oncogenes for indefinite cellular proliferation. This observation is reflected in cell line establishment of $p53^{-/-}$/CD2-myc^{+} and $p53^{-/-}$ lymphoma cells where $p53^{-/-}$/CD2-myc^{+} cells, expressing the CD2-*myc* transgene, consistently established in culture but only a proportion of $p53^{-/-}$ tumours did. It should be noted however that the $p53^{-/-}$ cells were derived from tumours and would be expected to have secondary mutations, possibly capable of complementing the $p53^{-/-}$ genotype in immortalisation. The results of Metz *et al.* indicated that the absence of p53 had a direct effect on cellular expansion and that immortalisation was not due to uncontrolled growth of cells which had sustained a mutation in a senescence gene.

3.3.5 Loss of p53-Dependent Apoptosis To Explain Synergy with c-*myc*.

Under certain conditions, the c-*myc* oncogene can induce apoptosis rather than proliferation (Evan *et al.*, 1992; Shi *et al.*, 1992; Askew *et al.*, 1991). The specific inhibition of c-*myc* mediated apoptosis by the *bcl*-2 oncogene may well have accounted for the potent synergy observed between these oncogenes (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992; Marin *et al.*, 1995). Loss of p53 associated apoptosis has been shown by Lowe and co-workers to directly co-operate with overexpression of the E1A oncogene in the induction and progression of tumour development *in vivo* (Lowe *et al.*, 1994a; Symonds *et al.*, 1994). Therefore it is possible that the collaboration observed between p53 loss and deregulated c-*myc* may occur as a result of attenuated apoptosis.

Several lines of evidence support the hypothesis that loss of p53-dependent apoptosis is important for the synergy observed between c-*myc* and $p53^{-/-}$ lesions. Firstly, Wang and co-workers observed that reconstitution of wild type p53 into two established cell lines deficient for p53 and overexpressing *myc* oncogenes resulted in rapid cell death by apoptosis (Wang *et al.*, 1993a; Ramqvist *et al.*, 1993). Furthermore, this p53-induced apoptosis was inhibited by overexpression of the *bcl*-2 oncogene (Wang *et al.*, 1993b). Lotem and Sachs (1993b) similarly found that overexpression of c-*myc* in M1 myeloid leukaemia cells under wild type p53 conditions induced apoptosis and that loss of p53 resulted in the suppression of apoptosis. These authors suggested that by abrogating the c-*myc* apoptotic pathway, loss of p53 would cause c-*myc* expression to drive cells into proliferation (Sachs and Lotem, 1993). In this way p53 loss would parallel the action of the *bcl*-2 oncogene (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992). This hypothesis was supported by two reports demonstrating that wild type p53 was directly required for the execution of the c-*myc* induced apoptotic pathway in fibroblasts, and that lack of p53 in these cells prevented c-*myc* associated cell death (Wagner *et al.*, 1994; Hermeking and Eick, 1994). In this respect, c-*myc* mediated apoptosis resembles E1A-induced apoptosis which also requires functional p53 protein (Lowe *et al.*, 1993b; Debbas and White, 1993; Lowe *et al.*, 1994a). While the combination of wild type p53 and overexpressed c-*myc* was found to induce apoptosis in rat hepatocellular carcinoma cells, expression of wild type p53 in the absence of c-*myc* expression did not induce apoptosis but rather mediated growth arrest (Saito and Ogawa, 1995). These authors concluded therefore that p53-mediated apoptosis was a specific response to overexpressed c-*myc* (Saito and Ogawa, 1995).

3.3.6 The Role of p53-Dependent Apoptosis in the $p53^{-/-}$/CD2-myc^{+} Model.

Cell lines derived from $p53^{-/-}$/CD2-myc^{+} lymphomas were found to uniformly establish in culture whereas CD2-*myc* cell lines consistently failed to do so. It could be postulated that this result reflects a blockage in the c-*myc* apoptotic pathway which is associated with loss of p53. In an attempt to discern the possibility that abrogation of p53-dependent apoptosis was important in the pathogenesis of the tumours arising in the $p53^{-/-}$/CD2-myc^{+} mice, tumour tissue was examined by TUNEL analysis. Preliminary

investigations have indicated that the degree of apoptosis in $p53^{-/-}$/CD2-*myc*$^{+}$ tumours was no different to that observed with both CD2-*myc* and $p53^{-/-}$ control tumours. These results are consistent with those of Hsu *et al.* (1995) who similarly used TUNEL analysis to demonstrate that there was no significant difference in apoptotic levels between Eμ-*myc* tumours with and without wild type p53. These authors suggested that the accelerated lymphoma development in $p53^{+/-}$/Eμ-*myc* tumours was not due to a reduction in apoptosis but rather to the loss of p53 growth arrest function, and that p53 was not required for c-*myc* mediated apoptosis during lymphomagenesis (Hsu *et al.*, 1995).

However, it should be noted that apoptosis does occur in $p53^{-/-}$ lymphomas as was previously described by Purdie *et al.* (1994). This was also observed in the $p53^{-/-}$ lymphomas analysed by TUNEL in the current study where apoptosis levels were comparable to that of CD2-*myc* and $p53^{-/-}$/CD2-*myc*$^{+}$ tumours. These results confirm that apoptosis occurs by p53-dependent and p53-independent pathways (Clarke *et al.*, 1993; Lowe *et al.*, 1993a). Indeed, it has been shown that c-*myc* induced apoptosis can occur by p53-dependent and p53-independent pathways (Sakamuro *et al.*, 1995). It would be expected therefore that $p53^{-/-}$/CD2-*myc*$^{+}$ cells would be capable of executing more than one apoptotic pathway. Moreover, it should be emphasized that the $p53^{-/-}$ /CD2-*myc*$^{+}$ tissues analysed in this study were tumours which may have sustained multiple genetic hits. Any one of these events may have influenced apoptotic programs. Consequently the apoptosis exhibited by $p53^{-/-}$/CD2-*myc*$^{+}$ tumours may not be specifically associated with c-*myc*. In conclusion therefore, although these results do not indicate that the $p53^{-/-}$ lesion acts to suppress CD2-*myc*-induced apoptosis in these tumours, neither do they discount this possibility. Further investigations with non-tumourous tissue would be required to formally address this issue.

As a tumour suppressor, wild type p53 can act as a cell cycle checkpoint monitoring the cell for damage and elicit growth arrest and/or apoptosis as required. It was perhaps not surprising then that loss of p53 collaborated so effectively with c-*myc* when one considers the consequence of abrogating any one or all of these functions. A subjective scenario to explain this interaction could be proposed. Stochastic activation of CD2-*myc* causes overexpression of the transgene in cells which survive due to the lack of a p53

cell cycle checkpoint. These *myc*-overexpressing cells are unable to mediate a p53-dependent c-*myc* apoptosis pathway and so continue to proliferate. A few clones of the expanded cell population undergo further mutations leading to the development of rapid, highly metastatic oligoclonal T-cell lymphomas. Further analyses with the $p53^{-/-}$/CD2-myc^{+} model may provide formal evidence to support part or all of this hypothetical mechanism.

CHAPTER 4.

A STUDY OF EARLY NEOPLASTIC CHANGES IN p53$^{-/-}$/CD2-*myc*$^{+}$ TRANSGENIC MICE.

4.1 INTRODUCTION

It was demonstrated in the preceding chapter that the CD2-*myc* transgene and p53$^{-/-}$ mutation co-operate to produce early onset T-cell lymphomas in transgenic mice which carry both of these lesions. The synergy observed in these animals may be due to changes in the initiation or the progression rates of tumour development. Although clinical signs of lymphomagenesis became apparent at between 60 and 86 days, it is not known when tumours were first initiated in these mice or whether pre-neoplastic disturbances occurred. It was important therefore to assess the course of tumourigenesis in these animals by investigating when tumours arose and how this related to the progression of disease.

The nature of the synergy between the CD2-*myc* and p53$^{-/-}$ lesions has not been established although a number of possible hypotheses have been put forward (as discussed in chapter 3). In order to understand the mechanism of synergy between these lesions it was necessary to first assess the role of the CD2-*myc* transgene in the pathogenesis of p53$^{-/-}$/CD2-*myc*$^{+}$ tumours. Studies of CD2-*myc* transgenic mice have found that although expression of the CD2-*myc* transgene is invariably associated with lymphoma, it is not detected in normal transgenic tissues (Stewart *et al.*, 1993). It is possible therefore that the lack of transgene expression in healthy tissues may explain the relatively low tumour incidence and lack of pre-lymphomatous disturbances in CD2-*myc* transgenic animals as compared to other *myc* transgenic models (Adams *et al.*, 1985; Langdon *et al.*, 1986; Schmidt *et al.*, 1988). CD2-*myc* transgenic mice on a p53$^{-/-}$ background developed tumours with 100% incidence which contrasts with the low tumour incidence of CD2-*myc* mice on a wild type p53 background. All p53$^{-/-}$/CD2-*myc*$^{+}$ lymphomas expressed the CD2-*myc* transgene confirming the involvement of CD2-*myc* in these tumours (section 3.2.5). It is possible therefore that one of the effects of p53 loss may be to permit constitutive expression, or early activation, of the CD2-*myc*

transgene, perhaps as a result of the genetic instability of p53 null cells. Frequent stochastic activation would not necessarily lead to constitutive expression of the transgene but would conceivably account for the increased frequency of CD2-*myc* expressing lymphomas.

It has been established that the c-*myc* oncogene induces apoptosis as well as cellular proliferation (Evan *et al.*, 1992) with the suggestion that p53 may be required for execution of this apoptotic pathway (Wagner *et al.*, 1994; Hermeking and Eick, 1994). Abrogation of *myc*-mediated apoptosis in p53 null cells may therefore result in the unscheduled survival of CD2-*myc* expressing cells. This would be concordant with previous studies which demonstrated that abrogation of oncogene-associated apoptosis by p53 loss directly influenced tumourigenesis in response to overexpression of E1A and SV40 large T oncogenes (Lowe *et al.*, 1994a; Symonds *et al.*, 1994). If the absence of p53 in $p53^{-/-}$/CD2-myc^{+} cells allows constitutive CD2-*myc* expression then it may be possible to detect pre-neoplastic disturbances in the tissues of $p53^{-/-}$/CD2-myc^{+} mice as was seen in young Eμ-*myc* transgenic animals (Langdon *et al.*, 1986).

In order to address the question of whether p53 loss leads to constitutive expression of the CD2-*myc* transgene and how this relates to tumour progression, young $p53^{-/-}$/CD2-myc^{+} and control animals were sacrificed at various time points prior to the time of clinical onset of lymphomagenesis. The tissues of these animals were subsequently examined for neoplastic changes and for CD2-*myc* transgene expression.

4.2 RESULTS.

4.2.1 Experimental Animals.

In order to generate $p53^{-/-}$/CD2-myc^{+} experimental animals and the necessary $p53^{-/-}$ and $p53^{+/+}$/CD2-myc^{+} parental controls, CD2-*myc* transgenic mice (Stewart *et al.*, 1993) were crossed with homozygous p53 null animals (Donehower *et al.*, 1992) to produce heterozygous animals, $p53^{+/-}$/CD2-myc^{+}, which were bred together or backcrossed with

p53 null mice. In addition to generating the relevant experimental genotypes, this second generation breeding program produced $p53^{+/-}$/CD2-*myc*$^{+}$, $p53^{+/-}$ and $p53^{+/+}$ littermates. A total of 196 animals were sacrificed at specific time intervals between 20 and 60 days of age (Table 4.1). Litters of animals were sampled and clinically examined without prior genotype knowledge to provide an unbiased assessment of pathological status.

Table 4.1 Experimental Animals Sacrificed Between 20 and 60 Days of Age.

Time Interval	Number of Mice Culled and Sampled					
	$p53^{-/-}$/CD2-*myc*$^{+}$	$p53^{-/-}$	$p53^{+/-}$/CD2-*myc*$^{+}$	$p53^{+/-}$	$p53^{+/+}$/CD2-*myc*$^{+}$	$p53^{+/+}$
60 days	6	6	13	10	5	1
51 days	1*					
50 days	6	1	19	4	10	2
49 days	1*					
40 days	6	4	13	5	5	3
30 days	7	-	9	7	5	2
27 days	3	-	2	9	4	1
20 days	2	1	12	7	3	1
Total No. of Mice Sampled	32	12	68	42	32	10

* One $p53^{-/-}$/CD2-*myc*$^{+}$ animal at each of 49 and 51 days had to be sacrificed in advance of the appointed time interval due to manifestation of ill health.

4.2.2 Tumour Development of $p53^{-/-}$/CD2-*myc*$^{+}$ Transgenic Mice Before 60 Days of Age.

More than 70% of $p53^{-/-}$/CD2-*myc*$^{+}$ mice (23/32) culled between 20 and 60 days of age were found to have lymphoblastic thymic lymphoma as assessed by histopathological

examination (Table 4.2). All $p53^{-/-}$/CD2-*myc*$^{+}$ animals culled at the ages of 60 days (6/6) and 49-51 days (8/8) were found to have pre-existing tumours (Figure 4.1). A high proportion of animals sacrificed at 40 days (3/6) and 30 days (5/7) were also found to harbour a thymic lymphoma as did 1 out of 3 animals culled at 27 days of age (Table 4.3 & Figure 4.1). These results demonstrate that lymphomas arise in young $p53^{-/-}$/CD2-*myc*$^{+}$ mice as early as post-natal day 27. No evidence of lymphomagenesis was found in two mice sacrificed at 20 days of age suggesting that the initiation of $p53^{-/-}$/CD2-*myc*$^{+}$ tumours may be between 20 and 30 days of age.

Table 4.2 Detection of Thymic Lymphomas in $p53^{-/-}$/CD2-*myc*$^{+}$ Transgenic Mice and Control Groups.

Time Interval	Number of Animals with Thymic Lymphoma#		
	$p53^{-/-}$/CD2-*myc*$^{+}$	$p53^{-/-}$	$p53^{+/+}$/CD2-*myc*$^{+}$
60 days	100% (6/6)	33% (2/6)	0% (0/5)
49-51 days	100% (8/8)	0% (0/1)	0% (0/10)
40 days	50% (3/6)	0% (0/4)	0% (0/5)
30 days	71% (5/7)	-	0% (0/5)
27 days	33% (1/3)	-	0% (0/4)
20 days	0% (0/2)	0% (0/1)	0% (0/3)

as determined by histopathological examination.

A number of control animals were also assessed for histological evidence of lymphomagenesis. Two out of 12 (17%) of the $p53^{-/-}$ parental controls were found to have a pre-existing lymphoma at the time of sampling (Table 4.2). Both of these animals were sacrificed at 60 days of age. None of the 32 $p53^{+/+}$/CD2-*myc*$^{+}$ controls had developed a thymic lymphoma (Table 4.2). Likewise, none of the $p53^{+/-}$ or $p53^{+/+}$ littermates showed evidence of lymphoma development however, one $p53^{+/-}$/CD2-*myc*$^{+}$ mouse (1/68), which was sacrificed at post-natal day 50, had a lymphoblastic thymic lymphoma with splenic involvement.

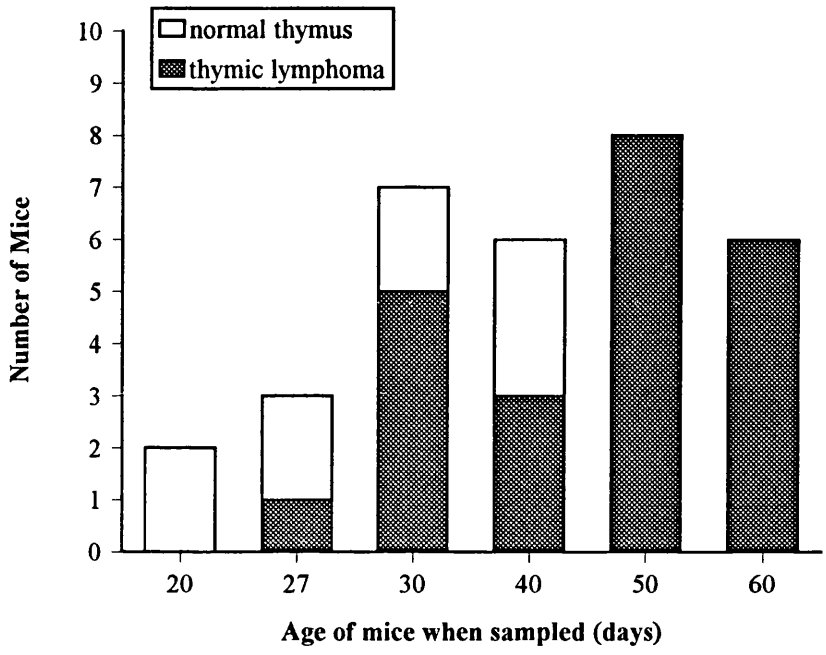

Figure 4.1. Development of Thymic Lymphoma in p53-/- CD2-myc+ Mice Between 20 and 60 Days of Age.

This figure illustrates the number of p53-/- CD2-myc+ animals which had histologically defined thymic lymphomas when sacrificed at appointed time intervals. Shaded boxes represent the number of mice which had thymic lymphoma and open boxes those which showed no obvious signs of lymphoma development at histopathological analysis. It is evident that a high percentage of p53-/- CD2-myc+ mice had developed thymic tumours at ages of between 30 and 60 days.

4.2.3 Clinical and Pathological Findings in Young p53$^{-/-}$/CD2-*myc*$^{+}$ Mice.

A number of criteria were used to assess the pathological status of p53$^{-/-}$/CD2-*myc*$^{+}$ animals sampled at various time intervals between 20 and 60 days of age. Based on clinical signs, gross pathology observations, histopathological examination and thymic weight, a range of pathologies were observed (Table 4.3).

A number of p53$^{-/-}$/CD2-*myc*$^{+}$ animals which showed abnormalities in all of the above criteria were found to be in advanced stages of disease progression. Seven of the 32 (22%) p53$^{-/-}$/CD2-*myc*$^{+}$ animals exhibited obvious clinical signs characterised by cachexia and tachypnoea. Clinical onset was observed in animals aged between 49 and 60 days (Table 4.3). It is not clear why a number of p53$^{-/-}$/CD2-*myc*$^{+}$ animals displayed overt clinical signs at times earlier than previous cohorts (chapter 3). This may be due to differences in genetic background, animal house conditions or increased cohort size. As expected, these animals displayed gross thymic enlargement and metastasis to secondary organs when examined at post-mortem. Thymic weight was clearly accentuated in these animals constituting between 2% and 9.5% of the total body weight as compared to an average of 0.6% for normal control thymus. Histopathological diagnosis confirmed that these animals had lymphoblastic thymic lymphoma and multiorgan involvement.

Histopathological examination of the tissues of p53$^{-/-}$/CD2-*myc*$^{+}$ animals revealed a spectrum of pathologies ranging from lymphoblastic thymic lymphoma to no detectable abnormality (Table 4.3). In many cases (16/32) the normal architecture of the thymus was totally replaced by lymphoblasts showing a high mitotic rate consistent with lymphoblastic thymic lymphoma (Figure 4.2). These lymphomas were frequently accompanied by metastatic spread to the spleen (12/16) and occasionally also showed lymphoblastic infiltration of other organs (5/16). Early, but obvious, stages in the progression of thymic lymphoma were observed in another 7 p53$^{-/-}$/CD2-*myc*$^{+}$ animals (Table 4.3). The normal architecture of the thymus was still recognisable in these tissues however the cortex, and occasionally the medulla, was partially replaced by islands of lymphoblasts (Figure 4.2). The degree of lymphoblastic infiltration in these tissues ranged from small focal accumulation of tumour cells to near total replacement of the

normal tissue. Infrequent metastatic spread (1/7) was observed in animals which exhibited these early stages of thyoma.

Post-mortem observations of $p53^{-/-}$/CD2-*myc*$^{+}$ mice often, but not always, reflected histopathological diagnosis. A number of animals which had histologically defined stages of thymic lymphoma displayed no obvious thymic enlargement at post-mortem (9 of 23). Many animals did however display obvious thymic enlargement and increased thymic weight at necropsy. The extent of thymic expansion varied from slight to gross enlargement and was related to age with animals sacrificed at between 50 and 60 days displaying a larger increase in thymus size than animals culled at an earlier age. It is noteworthy that one $p53^{-/-}$/CD2-*myc*$^{+}$ animal which had an obviously enlarged thymus at necropsy, exhibiting a higher than average thymic weight, did not have histological evidence of lymphomagenesis. It may be possible that the section of tissue which was analysed by microscopy was free of tumour cells in which case histological diagnosis may have underestimated the total number of animals in this study with lymphoma.

There was no obvious evidence to suggest that pre-lymphomatous disturbances occurred in young $p53^{-/-}$/CD2-*myc*$^{+}$ mice. Three $p53^{-/-}$/CD2-*myc*$^{+}$ animals, culled between the ages of 27 and 40 days of age, did have an increased rate of mitotic activity. However as several control tissues were also described as displaying an increased mitotic rate, it is probable that this feature was not significant with respect to tumourigenesis. One $p53^{-/-}$ /CD2-*myc*$^{+}$ mouse (p60pm154), which was sacrificed at 30 days of age, was found to exhibit possible pre-lymphomatous changes as assessed by microscopic examination. The thymic cortex of this mouse was populated by polymorphic small dark lymphocytes and large pale blast cells, which may have represented early tumour development. The significance of this finding was unclear however as the presence of blast cells was occasionally seen in the thymic tissues of $p53^{+/+}$/CD2-*myc*$^{+}$ and $p53^{-/-}$ mice suggesting it may represent an incidental finding.

Table 4.3 Summary of Criteria used to Assess Clinical and Pathological Status of Young p53$^{-/-}$/CD2-*myc*$^{+}$ Mice.

Clinical & Pathological Observations	No. of Animals at each Time Interval						Total No. of Animals
	60 days (n=6)	49 - 51 days (n=8)	40 days (n=6)	30 days (n=7)	27 days (n=3)	20 days (n=2)	
Clinical Signs							
Overt ill health	2	5	0	0	0	0	7
No obvious illness	4	3	6	7	3	2	25
Histopathology of Thymus							
Lymphoblastic thymic lymphoma	4	8	2	1	1	0	16
Early, but obvious, thymic lymphoma	2	0	1	4	0	0	7
Prelymphomatous changes? (see text)	0	0	0	1	0	0	1
Increased mitotic rate	0	0	2	0	1	0	3
No abnormality	0	0	1	1	1	2	5
Metastasis (based on histopathology)							
Multiorgan	1	4	0	0	0	0	5
Spleen only	2	3	1	2	0	0	8
None observed	3	1	5	5	3	2	19
Gross Pathology of Thymus							
Obvious enlargement	4	7	4	2	0	0	17
Normal size	2	1	2	5	3	2	15
*% Thymic Weight**							
Above average	3	7	2	2	0	1	15
Within normal range	3	1	4	5	3	1	17

* % thymus weight to total body weight of animal. Normal range was based on age-matched control littermates.

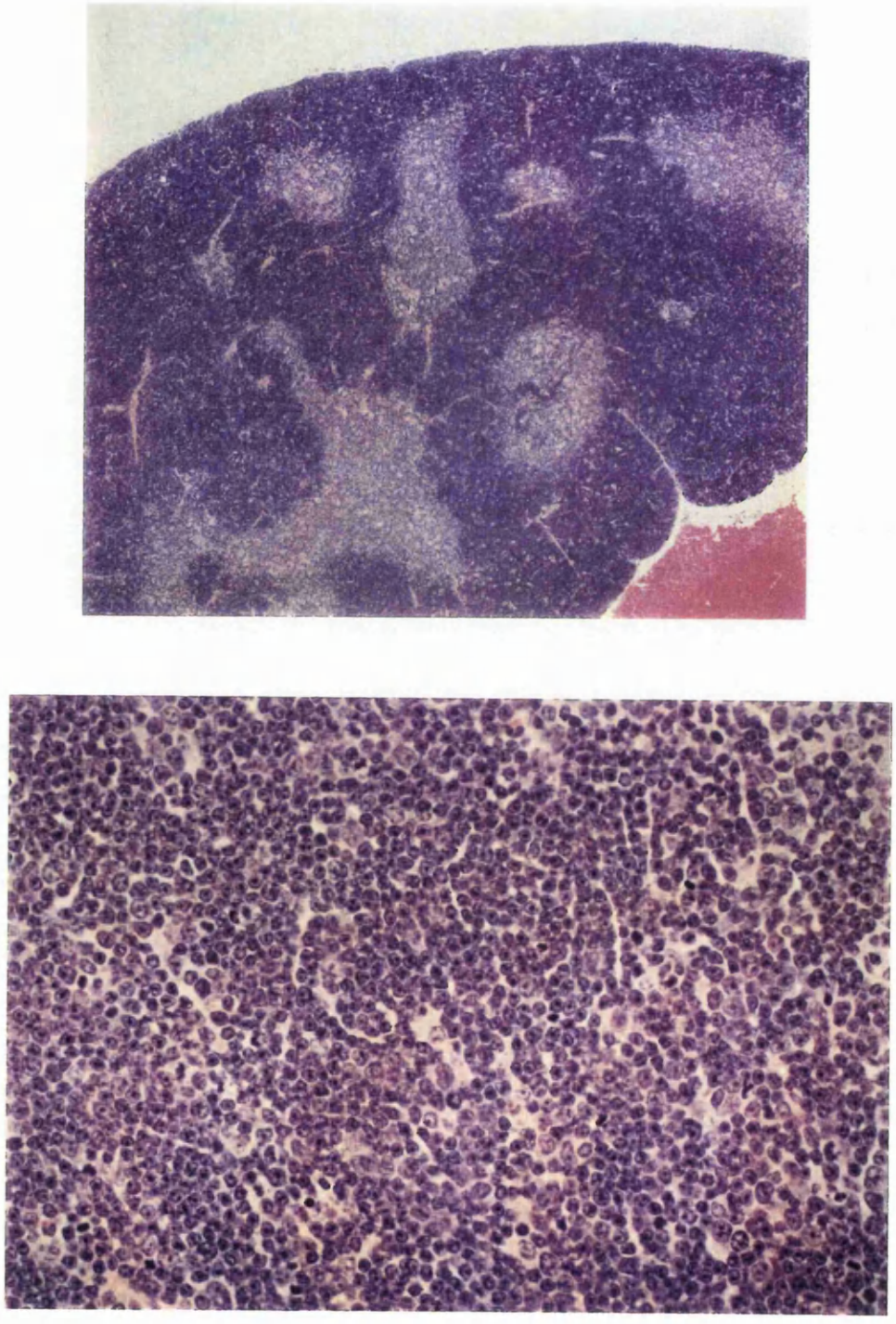

Figure 4.2.a. Normal Mouse Thymus.

Haematoxylin and eosin (H&E) stained normal mouse thymus. (Original in colour).
Top: This figure demonstrates the normal thymic architecture showing the pale coloured medulla and darker staining, denser cortex (H&E, x10). Bottom: The uniformity of the darker staining, small cortex cells can be seen at the higher magnification of mouse thymus (H&E, x100).

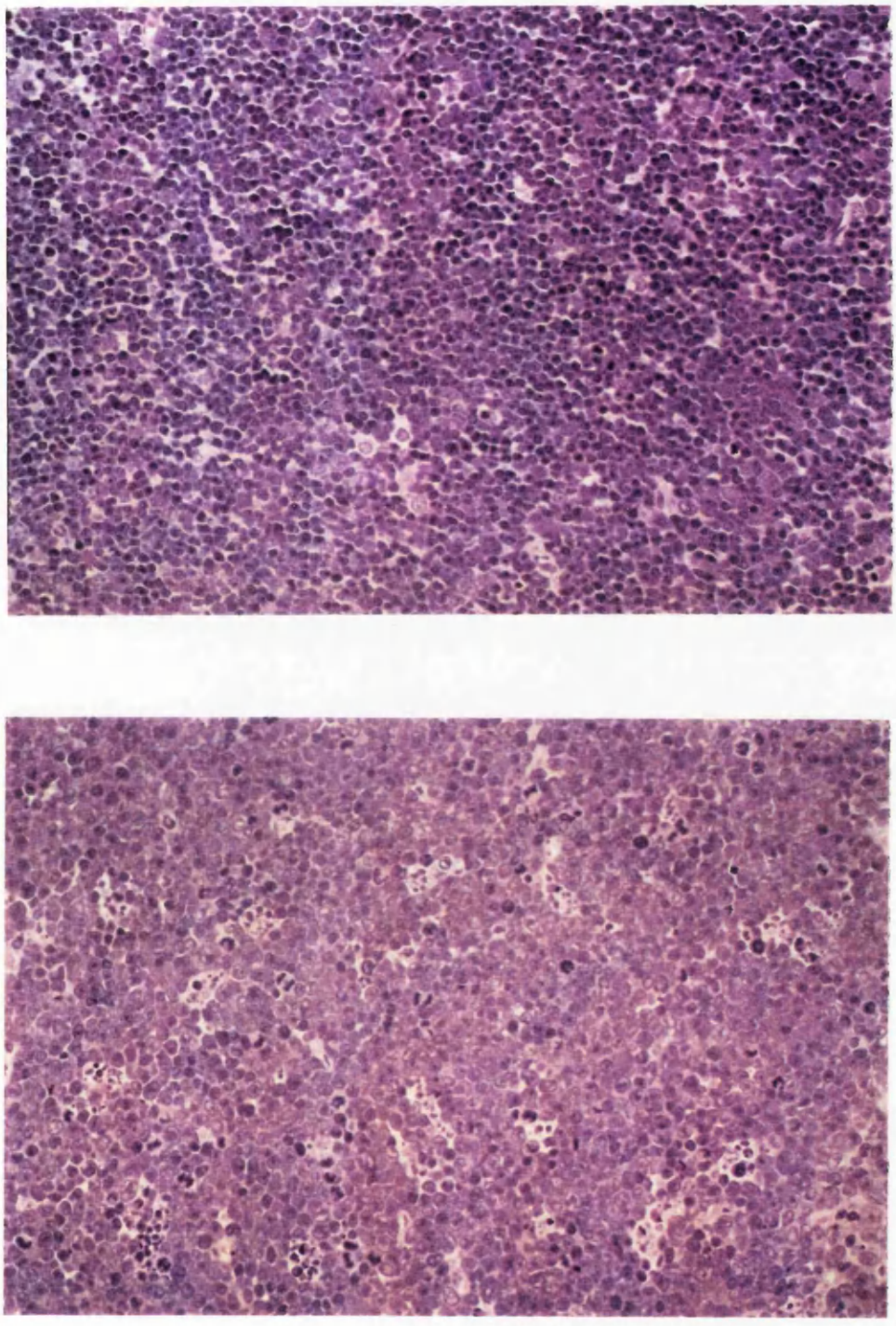

Figure 4.2.b. Thymus Tissue of Young p53-/- CD2-myc+ Mice; Normal and Lymphoma.

Top: Thymus of a young p53-/- CD2-myc+ mouse which showed no histopathological abnormalities (H&E, x100). Bottom: Thymus of a young p53-/- CD2-myc+ mouse which had a thymic lymphoma. The normal thymus cells have been replaced by large, pale staining lymphoblasts showing a high mitotic rate (H&E, x100). (Original in colour).

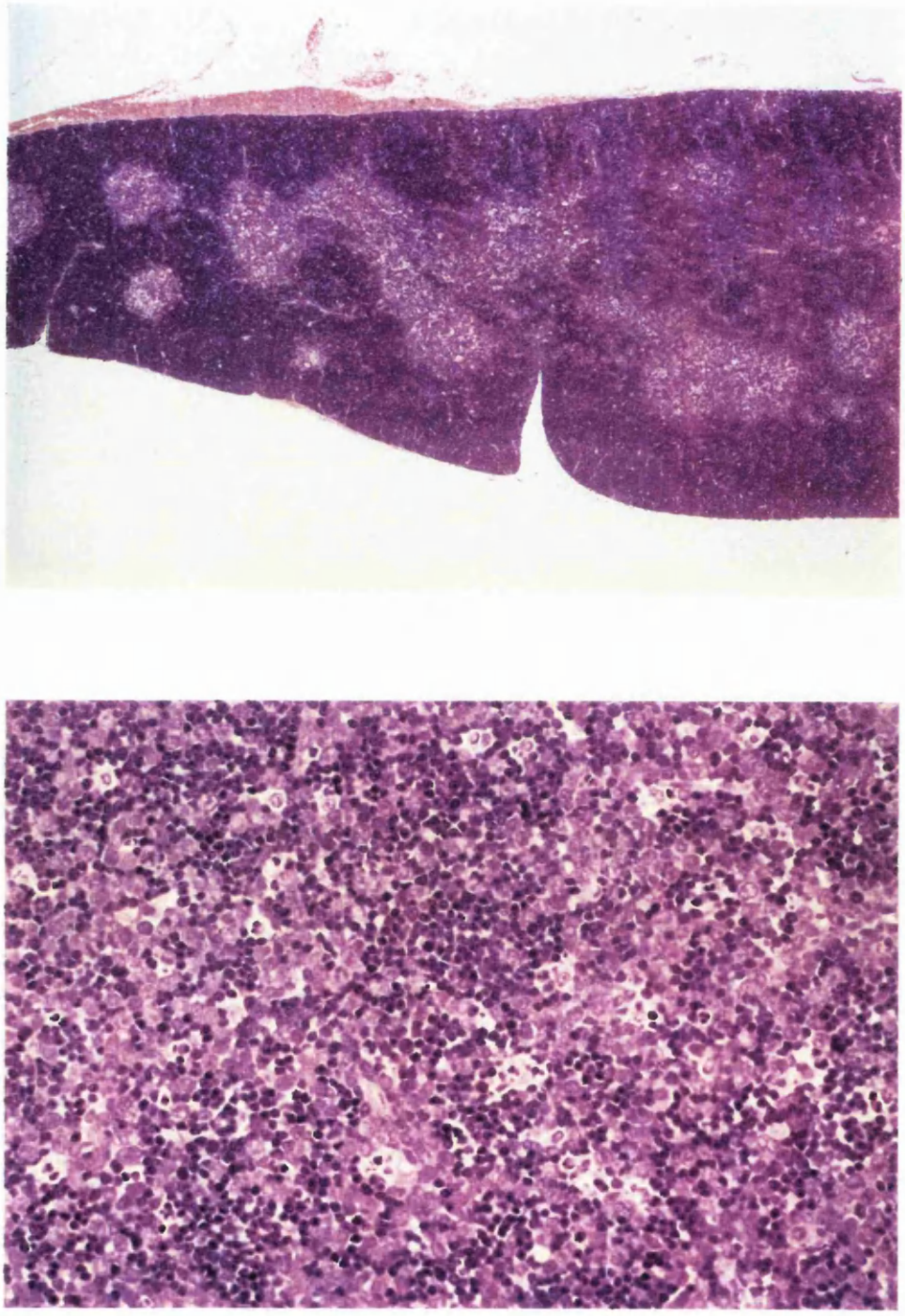

Figure 4.2.c. Early Stages of Thymic Lymphoma Development in p53-/- CD2-myc+ Mice.

This figure illustrates an early stage in the progression of a thymic lymphoma in a young p53-/- CD2-myc+ mouse. (Original in colour). Top: At low magnification the left hand side of the thymus appears normal showing cortex and medullary regions, while to the right of the photograph replacement of normal cells with large pale staining lymphoblasts can be seen (H&E, x10). Bottom: High magnification of early stage thymic tumour with islands of lymphoblast cells partially replacing the small, dark normal thymus cells (H&E, x100).

4.2.4 CD2-*myc* Transgene Expression.

Northern blot analysis revealed that histologically normal $p53^{-/-}$/CD2-myc^{+} thymic tissue did not express CD2-*myc* transgene mRNA (Figure 4.3). In contrast, all of the $p53^{-/-}$/CD2-myc^{+} thymic lymphomas which were examined did express CD2-*myc* mRNA. These results demonstrate that the pattern of transgene expression in $p53^{-/-}$/CD2-myc^{+} tissues is comparable to that of CD2-*myc* transgenic mice (Stewart *et al.*, 1993) and that loss of p53 does not result in constitutive expression of the transgene. CD2-*myc* expression was closely correlated with lymphoma development in $p53^{-/-}$/CD2-myc^{+} animals as tissues with early stages of thymic lymphoma development were found to express transgene mRNA (Figure 4.3). Importantly, the $p53^{-/-}$/CD2-myc^{+} mouse described above which exhibited possible pre-lymphomatous changes at 30 days of age (p60pm154), showed detectable levels of CD2-*myc* mRNA (Figure 4.3). No expression was detected in tissues which had showed an increased mitotic rate. Two $p53^{+/+}$/CD2-myc^{+} control mice which had displayed possible early neoplastic changes, characterised by an expanded cortex and a high mitotic rate, showed no detectable expression of the transgene.

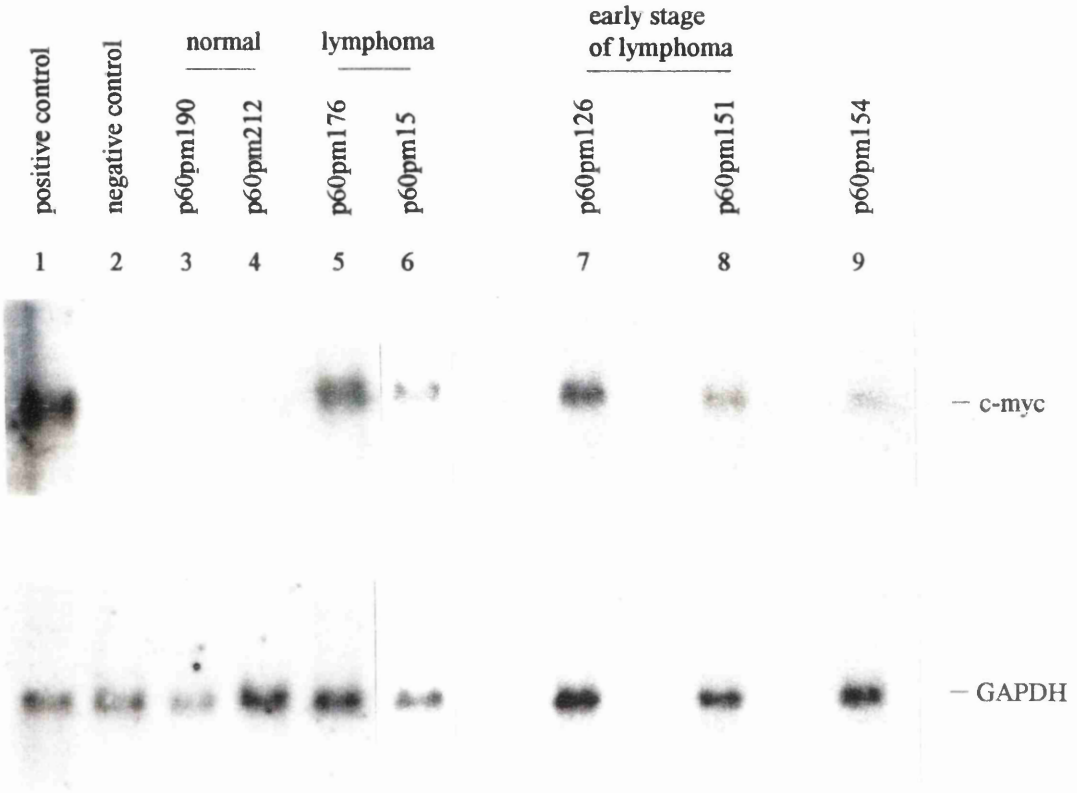

Figure 4.3. Northern Blot Analysis of Thymus Tissue of Young p53-/- CD2-myc+ Mice.

Thymus samples of young p53-/- CD2-myc+ mice were examined for CD2-myc transgene expression using Northern blot analysis. Lanes 1 and 2 are positive and negative controls respectively. Lanes 3 and 4 represent histologically normal p53-/- CD2-myc+ thymus tissue. Lanes 5 and 6 were malignant thymic lymphomas. Lanes 7 and 8 represent thymus tissue which was in the early stages of thymic lymphoma. Lane 9 was a mouse (p60pm154) which displayed histological changes without obvious neoplastic development. Samples were also assessed for expression of the murine GAPDH gene to confirm the presence of RNA in the lanes.

4.3 DISCUSSION.

4.3.1 Thymic Lymphomas are Detected in Young p53$^{-/-}$/CD2-*myc*$^{+}$ Mice from Post-Natal Day 27.

Clinical onset of lymphomagenesis in p53$^{-/-}$/CD2-*myc*$^{+}$ animals was previously shown to present at between 60 and 86 days of age. To examine at what age these lymphomas first arose, litters of p53$^{-/-}$/CD2-*myc*$^{+}$ and control animals were sacrificed at various time intervals prior to the expected time of clinical onset. A large proportion of p53$^{-/-}$/CD2-*myc*$^{+}$ animals (72%) sacrificed between 20 and 60 days of age were found to have pre-existing thymic lymphomas as assessed by histopathological analysis. By contrast only 3/164 (2%) of littermate controls showed histological evidence of lymphoblastic lymphoma. Tumours were detected in p53$^{-/-}$/CD2-*myc*$^{+}$ transgenic mice as early as post-natal day 27, often without apparent clinical signs or overall thymic enlargement. Manifestation of clinical onset was found to correlate with advanced stages of disease involving gross pathological enlargement of the thymus and metastatic spread. Detection of lymphomas as early as 27 days of age suggested that these tumours could pre-exist in animals for at least a further 22 days before causing apparent distress to the animal. It is likely that additional genetic events take place during this latency period although no direct evidence is available to support this hypothesis. No evidence of tumour development was observed in animals sacrificed at 20 days of age. It could be proposed from these results that tumours probably arise in p53$^{-/-}$/CD2-*myc*$^{+}$ mice between 20 and 30 days of age. It should be noted nevertheless that these studies cannot rule out the possibility that 20 day old animals did have small tumours which were not detected by the methods applied. Furthermore, a definite conclusion cannot be drawn on the analysis of such a small sample size of 20 day mice.

4.3.2 Early Neoplastic Changes and the Role of the CD2-*myc* Transgene.

The thymus of one p53$^{-/-}$/CD2-*myc*$^{+}$ animal (p60pm154) which was sacrificed at day 30 showed signs to indicate possible early neoplastic changes with the abnormal presence of polymorphic small dark and large pale lymphoid cells in the cortex. Expression of the

CD2-*myc* transgene in the thymic tissue of p60pm154 was consistent with the idea that these changes reflected an early stage in the pathogenesis of $p53^{-/-}$/CD2-myc^{+} tumours. Apart from this observation, no consistent pattern of pre-neoplastic changes was recorded in the tissues of $p53^{-/-}$/CD2-myc^{+} mice. An increased mitotic rate was displayed in the thymus of a number of $p53^{-/-}$/CD2-myc^{+} animals, however control mice also exhibited this feature which suggested that this histological finding was unlikely to be relevant to tumour development. Northern blot analysis failed to detect transgene expression in the thymic tissues of $p53^{-/-}$/CD2-myc^{+} mice which had increased mitotic activity.

Lack of detectable CD2-*myc* expression in normal tissues indicated that the CD2-*myc* transgene was not consistently expressed in $p53^{-/-}$/CD2-myc^{+} tissues, which presumably explains the absence of a pre-neoplastic phenotype. Nonetheless, expression of the transgene was found to correlate with lymphoma development in the double transgenic mice. The pattern of transgene expression in $p53^{-/-}$/CD2-myc^{+} mice was therefore equivalent to that of parental CD2-*myc* mice (Stewart *et al.*, 1993).

4.3.3 Implications for the Synergy Between CD2-*myc* and $p53^{-/-}$.

It can be concluded from these studies that lack of functional p53 did not result in constitutive CD2-*myc* expression in the thymic tissue of $p53^{-/-}$/CD2-myc^{+} animals. This result has implications for the proposed mechanisms by which p53 loss cooperates with the CD2-*myc* transgene. It had been suggested previously (chapter 3) that the CD2-*myc* transgene may be transcriptionally down-regulated by wild type p53, however this now seems highly improbable as the pattern of CD2-*myc* expression in $p53^{-/-}$/CD2-myc^{+} animals was no different to that of $p53^{+/+}$/CD2-myc^{+} animals. Lack of constitutive expression is also inconsistent with the theory that p53 loss rescues cells from apoptosis caused by an early phase of transgene expression in CD2-*myc* animals.

A more plausible explanation is that CD2-*myc* transgene expression occurs as a result of stochastic activation throughout the life of the CD2-*myc* mouse, but that sustained expression is dependent on other cellular events. The absence of p53 in $p53^{-/-}$/CD2-myc^{+}

cells evidently increases the rate of activation and/or provides an environment capable of sustaining transgene expression. Frequent, or premature, stochastic activation of the transgene may occur as a result of genetic instability created by the loss of a p53 cell cycle checkpoint (Lane, 1992). The absence of p53 in this way would affect the rate of tumour initiation in CD2-*myc* animals. This is consistent with the observation that all $p53^{-/-}$/CD2-myc^{+} animals developed tumours by 50 days whereas none of the $p53^{+/+}$/CD2-myc^{+} controls did. Previous studies have suggested that loss of p53-dependent apoptosis is an important determinant for the transformation of oncogene-expressing cells (Symonds *et al.*, 1994; Lowe *et al.*, 1994a). Sustained proliferation of CD2-*myc* expressing cells may therefore occur as a result of attenuated p53-dependent, *myc*-mediated apoptosis. However, if a block in apoptosis is the only mechanism by which p53 loss contributes to lymphoma development it would suggest that stochastic activation of the transgene is a relatively common event which is not entirely consistent with the lack of transgene expression in preneoplastic tissues. Furthermore, it was apparent from the results described previously (section 3.3.10) that the level of apoptosis in $p53^{-/-}$/CD2-myc^{+} tumours was not significantly reduced compared to the $p53^{+/+}$/CD2-myc^{+} tumours which suggested that inhibition of apoptosis had not obviously contributed to the pathogenesis of the double transgenic tumours.

It is apparent that loss of p53 affects the rate of initiation of lymphoma development in the $p53^{-/-}$/CD2-myc^{+} transgenic model. However, functional loss of p53 may also affect the progression rate of these tumours. Evidence suggests that tumours arise at around post-natal days 20-30, yet a number of animals developed highly malignant lymphoma and showed overt clinical signs at 50-60 days of age. It is possible that attenuated apoptosis, while not affecting tumour initiation, may influence the rate of tumour progression in these mice by permitting survival of proliferating cells. This would be consistent with the studies of Symonds and co-workers who reported that abrogation of p53-dependent apoptosis in transgenic mice expressing the SV40 large T antigen resulted in rapid and aggressive tumour progression but did not affect tumour initiation (Symonds *et al.*, 1994). TUNEL analysis of preneoplastic $p53^{-/-}$/CD2-myc^{+} tissues from young mice may discern whether reduced apoptosis is important for tumour progression in the $p53^{-/-}$/CD2-myc^{+} model. Analysis of early neoplastic stages differs from the

analysis carried out previously with tumour tissues (chapter 3) as apoptosis would be assessed prior to the time in which multiple genetic event presumably occur. In this way an assessment of apoptosis levels influenced by the $p53^{-/-}$ and CD2-*myc* lesions alone can be made.

CHAPTER 5.

TUMOUR DEVELOPMENT IN CD2-*MYC* TRANSGENIC MICE HETEROZYGOUS FOR A P53 NULL MUTATION.

5.1 INTRODUCTION.

The synergistic interaction between the CD2-*myc* transgene and homozygous p53 null mutation was demonstrated by the results presented in chapters 3 and 4. To continue this study, the combined effects of the CD2-*myc* transgene with the heterozygous p53 mutation were examined. Mice genotypically heterozygous for the p53 null mutation, hereafter referred to as heterozyotes or $p53^{+/-}$, differ from homozygous p53 null mice ($p53^{-/-}$) in that they carry a single wild type p53 allele and therefore produce functional p53 protein (Donehower *et al.*, 1992). Heterozygote animals were found to remain healthy for a longer period of time than homozygous null animals but succumbed to spontaneous tumour development between nine and eighteen months of age (Harvey *et al.*, 1993a). Genetic background may influence the tumour latency of p53 heterozygote animals as Purdie *et al.* (1994) reported that a significant proportion of the heterozygote animals in their study developed tumours before nine months of age. The rate of tumour development in homozygous null mice was similarly shown to be altered by genetic background (Harvey *et al.*, 1993b). Tumours which develop in heterozygote animals are frequently associated with loss of the remaining wild type p53 allele (Harvey *et al.*, 1993a; Purdie *et al.*, 1994; Jacks *et al.*, 1994). These tumours are effectively homozygous null for the p53 protein. The loss of wild type allele in the murine tumours reflects the loss of heterozygosity often observed in human tumours (Nigro *et al.*, 1989; Baker *et al.*, 1989; Malkin *et al.*, 1990; Levine *et al.*, 1991).

The results presented in this chapter describe a transgenic model designed to assess the consequence of the $p53^{+/-}$ mutation in animals carrying the CD2-*myc* transgene. This study also considers the status of the wild type p53 allele in the tumours of $p53^{+/-}$/CD2-myc^{+} mice to investigate if loss of the remaining wild type allele is important for the progression of CD2-*myc* induced lymphomas.

5.2 RESULTS.

5.2.1 Experimental Animals.

Transgenic mice heterozygous for both the p53 null mutation and CD2-*myc* transgene ($p53^{+/-}$/CD2-myc^{+}) were generated by breeding CD2-*myc* transgenic and homozygous p53 null animals (Stewart *et al.*, 1993; Donehower *et al.*, 1992). An experimental cohort of $p53^{+/-}$/CD2-myc^{+} mice (n=34), in addition to parental controls $p53^{+/+}$/CD2-myc^{+} (n=10) and $p53^{+/-}$ (n=24), were generated by the breeding program as previously outlined in chapter 3 (Table 3.1). These animals were monitored over an eighteen month period for signs of tumour development.

5.2.2 Tumour Development In Control Groups.

The survival and tumour incidence of parental controls was similar to that previously reported. CD2-*myc* transgenic mice develop tumours at a relatively low incidence, usually between 3 and 12 months of age (Stewart *et al.*, 1993). These mice develop exclusively T-cell lymphomas. Conditional expression of the CD2-*myc* transgene in neoplastic but not healthy tissues has linked the transgene with the pathogenesis of tumours arising in these mice (Stewart *et al.*, 1993). In accordance with the results of Stewart *et al.*, 30% (3/10) of the $p53^{+/+}$/CD2-myc^{+} control mice in this study developed lymphomas at between 4 and 8 months of age (Figure 5.1). One $p53^{+/+}$/CD2-myc^{+} mouse developed a renal cell carcinoma at 13 months, a tumour type not commonly observed in CD2-*myc* transgenic mice. This tumour was examined by Northern blot analysis and was found not to express transgene mRNA (Figure 5.2). It is likely therefore, that the development of this tumour was incidental and unrelated to the CD2-*myc* transgene.

As demonstrated by Figure 5.1, 75% (18/24) of $p53^{+/-}$ control mice developed tumours, with clinical signs occurring at between 9 and 18 months of age. Consistent with the observations of other authors (Harvey *et al.*, 1993a; Purdie *et al.*, 1994; Jacks *et al.*, 1994), tumours arising in the $p53^{+/-}$ mice were predominantly non-lymphoid (Table 5.1).

Sarcomas occurred in about 39% (7/18) of the tumour bearing $p53^{+/-}$ animals and carcinomas in approximately 22% (4/18). 39% (7/18) of animals which succumbed to tumourigenesis developed lymphoma. Two $p53^{+/-}$ mice presented with multiple tumours, one mouse had a co-existing liver cell carcinoma and lung adenocarcinoma and another mouse had fibrosarcomas at two different anatomical sites. Wild type control littermates ($p53^{+/+}$) did not develop tumours within the eighteen month observation period (n=10).

5.2.3 Tumour Development in $p53^{+/-}$/CD2-*myc*$^{+}$ Mice.

Survival and tumour incidence of mice which carried both the CD2-*myc* and $p53^{+/-}$ genetic lesions ($p53^{+/-}$/CD2-*myc*$^{+}$) are illustrated in Figure 5.1. Although the latency period was reduced for a proportion of $p53^{+/-}$/CD2-*myc*$^{+}$ mice, there was no difference in the end survival of $p53^{+/-}$/CD2-*myc*$^{+}$ animals compared to $p53^{+/-}$ littermates. Over the eighteen month period, $p53^{+/-}$/CD2-*myc*$^{+}$ and $p53^{+/-}$ cohorts were equally susceptible to tumour development with 76% (26/34) of $p53^{+/-}$/CD2-*myc*$^{+}$ mice and 75% (18/24) of $p53^{+/-}$ controls developing tumours. The $p53^{+/-}$/CD2-*myc*$^{+}$ animals were found to succumb to a variety of tumour types, the range of tumours being similar to that observed in the $p53^{+/-}$ group (Table 5.1). The most common tumour type observed in the $p53^{+/-}$/CD2-*myc*$^{+}$ mice was lymphoma with 62% (16/26) of tumour bearing mice developing lymphoblastic lymphoma. Sarcomas, predominantly fibrosarcomas and osteosarcomas, occurred in approximately 27% (7/26) of animals which developed tumours and adenocarcinomas in about 19% (5/26). It should be noted that these figures represent tumours per tumour bearing mice and animals which developed multiple tumour types have been included more than once. Two $p53^{+/-}$/CD2-*myc*$^{+}$ mice had more than one tumour type at post-mortem (Table 5.1). One mouse had a co-existing lymphoma and fibrosarcoma while a second animal had a co-existing osteosarcoma and tubular adenocarcinoma.

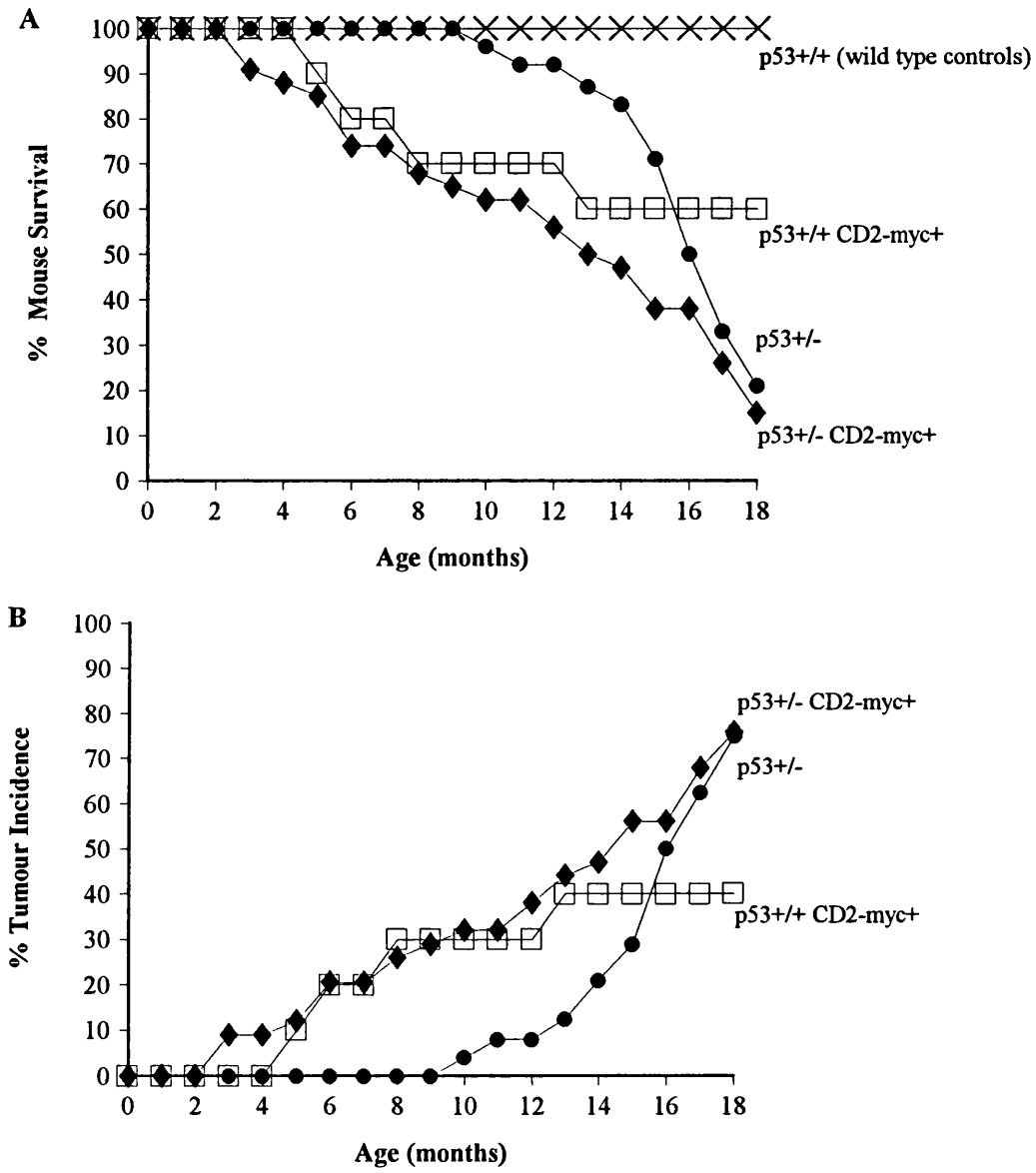

Figure 5.1. Survival and Tumour Incidence of p53+/- CD2-myc+ Mice and Littermate Controls.

A) Comparing the overall survival rates of p53+/- CD2-myc+ (◆, n=34), p53+/- (●, n=24), p53+/+ CD2-myc+ (□, n=10) and p53+/+ mice (X, n=10) over an eighteen month observation period. Mice were humanely culled when showing abnormal clinical signs. B) Percentage of animals from each of these groups which succumbed to obvious tumour development.

Table 5.1 Tumour Development in p53$^{+/-}$/CD2-*myc*$^{+}$ and p53$^{+/-}$ Mice.

A. Tumour Types of p53$^{+/-}$/CD2-*myc*$^{+}$ Mice.

Tumour Type	Incidence of Tumour Bearing Mice
Lymphoma	15/26 (58%)
Osteosarcoma	2/26 (7.5%)
Fibrosarcoma	2/26 (7.5%)
Tubular adenocarcinoma	2/26 (7.5%)
Lung adenocarcinoma	2/26 (7.5%)
Spindle cell tumour	1/26 (4%)
Tubular adenocarcinoma & osteosarcoma	1/26 (4%)
Fibrosarcoma & lymphoma	1/26 (4%)

B. Tumour Types of p53$^{+/-}$ Littermate Controls.

Tumour Type	Incidence of Tumour Bearing Mice.
Lymphoma	7/18 (39%)
Osteosarcoma	3/18 (17%)
Fibrosarcoma(s)	2/18 (11%)
Mammary adenocarcinoma	1/18 (5.5%)
Tubular adenocarcinoma	1/18 (5.5%)
Lung adenocarcinoma	1/18 (5.5%)
Undifferentiated sarcoma	1/18 (5.5%)
Leiomyosarcoma	1/18 (5.5%)
Liver cell carcinoma & lung adenocarcinoma	1/18 (5.5%)

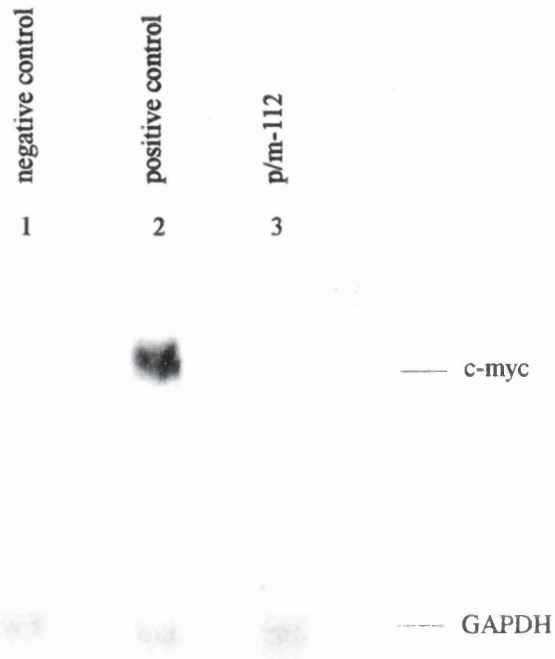

Figure 5.2. Northern Blot Analysis of p53+/+ CD2-myc+ Renal Cell Carcinoma.

The renal cell carcinoma which developed in a p53+/+ CD2-myc+ mouse at 13 months of age was examined for expression of the CD2-myc transgene to determine if the transgene had contributed to the pathogenesis of this tumour. Lane 1 is a CD2-myc negative control, lane 2 is a CD2-myc expressing tumour as a positive control and lane 3 is the p53+/+ CD2-myc+ renal cell carcinoma, the tumour name is shown above the lane number. The blot was also assessed for GAPDH expression to confirm the presence of RNA in each lane.

In addition to the 26 $p53^{+/-}$/CD2-*myc*$^{+}$ mice which developed obvious tumours, gross pathology and histopathology failed to identify a reason for ill health in two other mice, while extensive autolysis prevented diagnosis on a third animal. It is possible that these mice had a neoplastic condition but that the tumours were not detected during routine pathological investigations. For example, one mouse which presented with slight paresis may have harboured an osteosarcoma, or similar lesion, compressing the spinal cord. This would be consistent with observations by Purdie *et al.* (1994) who reported the presence of previously unidentified osteosarcomas in $p53^{+/-}$ animals with the use of skeletal x-rays.

A higher percentage of $p53^{+/-}$/CD2-*myc*$^{+}$ mice developed lymphomas compared to $p53^{+/-}$ parental controls, 62% versus 39%. These lymphomas involved the thymus and had varying degrees of metastatic spread to other lymphoid tissues. The pathology of these lymphomas was not dissimilar to that observed for both $p53^{+/-}$ and $p53^{+/+}$/CD2-*myc*$^{+}$ control mice. Immunophenotype analysis was carried out on $p53^{+/-}$/CD2-*myc*$^{+}$ lymphomas to determine the cell type of these tumours. With one exception, all lymphomas stained positive for at least one of the T-cell surface markers, CD4, CD8 or CD3. None of the tumours were found to be strongly positive for the B-cell marker CD45R (B220), although one lymph node tumour did have a small population of $B220^{+}CD3^{+}$ cells. The most common phenotype of the $p53^{+/-}$/CD2-*myc*$^{+}$ lymphomas was an immature $CD4^{+}CD8^{+}$ double-positive phenotype occurring in 8 out of 14 tumours (Table 5.2). $CD4^{+}CD8^{+}$ phenotypes are frequently displayed by CD2-*myc* and $p53^{+/-}$ lymphomas (Stewart *et al.*, 1993; Purdie *et al.*, 1994). One of the early $p53^{+/-}$/CD2-*myc*$^{+}$ lymphomas (p/m-28) had a $CD4^{-}CD8^{+}$ single-positive phenotype similar to that observed in early CD2-*myc* tumours (Cameron *et al.*, 1996).

5.2.4 Tumour Development in $p53^{+/-}$/CD2-*myc*$^{+}$ Mice May Reflect Additive CD2-*myc* and $p53^{+/-}$ Pathologies.

While $p53^{+/-}$ mice do not generally succumb to tumour development until after nine months of age, it was observed that a small proportion (29%) of $p53^{+/-}$/CD2-*myc*$^{+}$ mice developed tumours between two and nine months (Figure 5.1). A 30% tumour incidence

was similarly displayed by $p53^{+/+}$/CD2-*myc*$^{+}$ control mice, occurring between two and nine months of age. It is possible therefore that the $p53^{+/-}$/CD2-*myc*$^{+}$ tumours which developed before nine months of age were due to the CD2-*myc* lesion and were unrelated to the $p53^{+/-}$ mutation. Furthermore, it could be postulated that tumour development in the cohort of $p53^{+/-}$/CD2-*myc*$^{+}$ mice over a period of eighteen months, occurred as a result of additive pathologies associated with independent CD2-*myc* and $p53^{+/-}$ events. A number of experiments and comparative studies were applied to investigate this possibility.

Tumour latency of $p53^{+/-}$/CD2-*myc*$^{+}$ mice was compared against each of the parental controls as illustrated by Figure 5.3. It was apparent that $p53^{+/-}$/CD2-*myc*$^{+}$ tumours which developed before nine months did not reflect that of $p53^{+/-}$ mice but were coincident with the latency of CD2-*myc* tumours. It should be noted that the CD2-*myc* tumours presented in Figure 5.3.C have been taken from a cohort (n=31) outwith the current study but are representative of the $p53^{+/+}$/CD2-*myc*$^{+}$ littermate controls (n=3) in this experiment. The majority of CD2-*myc* tumours (including the $p53^{+/+}$/CD2-*myc*$^{+}$ tumours of the current study) arose between three and ten months of age. Although large numbers of CD2-*myc* transgenic mice have not been kept for long periods of time, very few of those kept beyond twelve months develop tumours. The late peak of $p53^{+/-}$/CD2-*myc*$^{+}$ tumours which occurred between eleven and eighteen months are therefore unlikely to represent CD2-*myc* related tumours. Rather, these tumours were characteristic of the tumour latency observed with $p53^{+/-}$ control littermates (Figure 5.3.B).

The spectrum of tumour types which $p53^{+/-}$/CD2-*myc*$^{+}$ mice developed was also compared to that of parental controls (Figure 5.4). All of the $p53^{+/-}$/CD2-*myc*$^{+}$ tumours occurring before ten months of age were lymphomas, analogous to the observed pathology in CD2-*myc* transgenic mice. In contrast tumours occurring after ten months of age were predominately non-lymphoid tumours. These tumour types were found to reflect the tumour spectrum observed with $p53^{+/-}$ mice and occurred with the same latency as the parental controls.

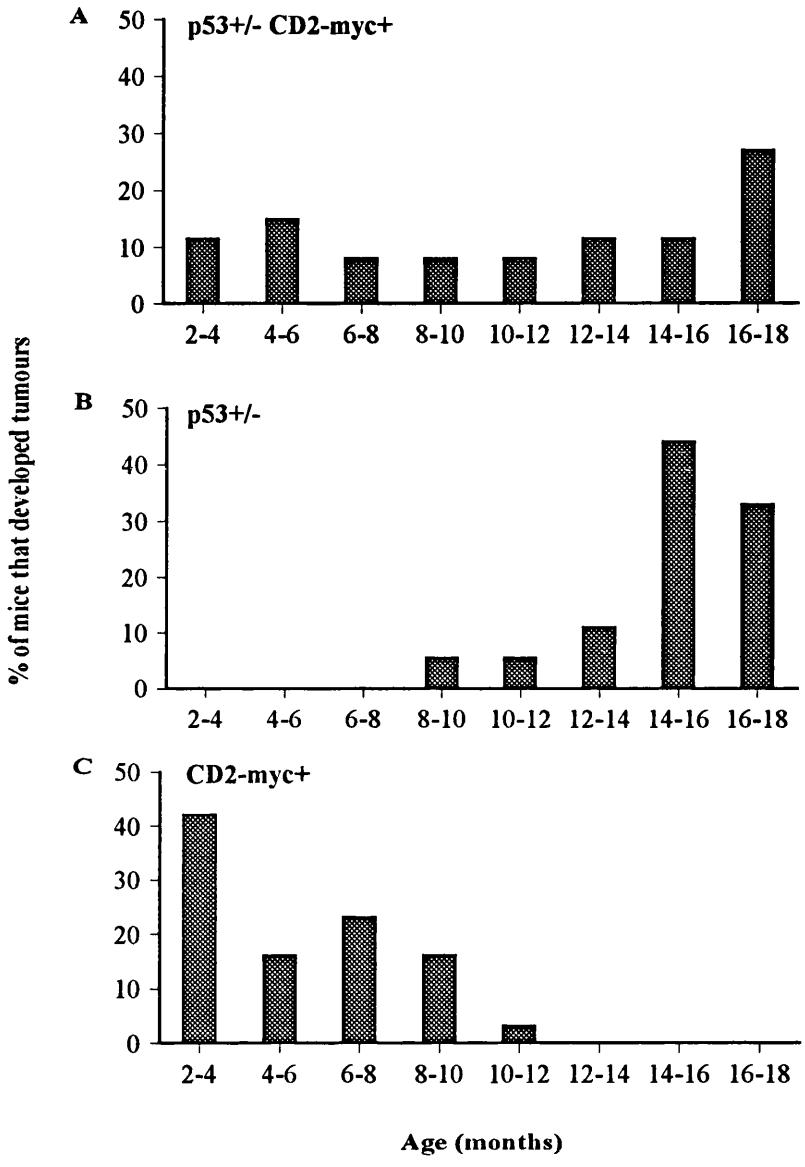

Figure 5.3. Latency of Tumour Development in p53+/- CD2-myc+ Mice and Parental Controls.

This figure illustrates the number of mice which developed tumours within a given time period expressed as a percentage of the number of mice in the cohort which developed tumours (n). A) p53+/- CD2-myc+ mice (n=26) are compared with B) p53+/- parental controls (n=18) and C) CD2-myc+ parental controls (n=31). It should be noted that due to the small number of p53+/+ CD2-myc+ tumours in the present study, a representative group of CD2-myc tumours outwith the current experimental group have been used for this figure.

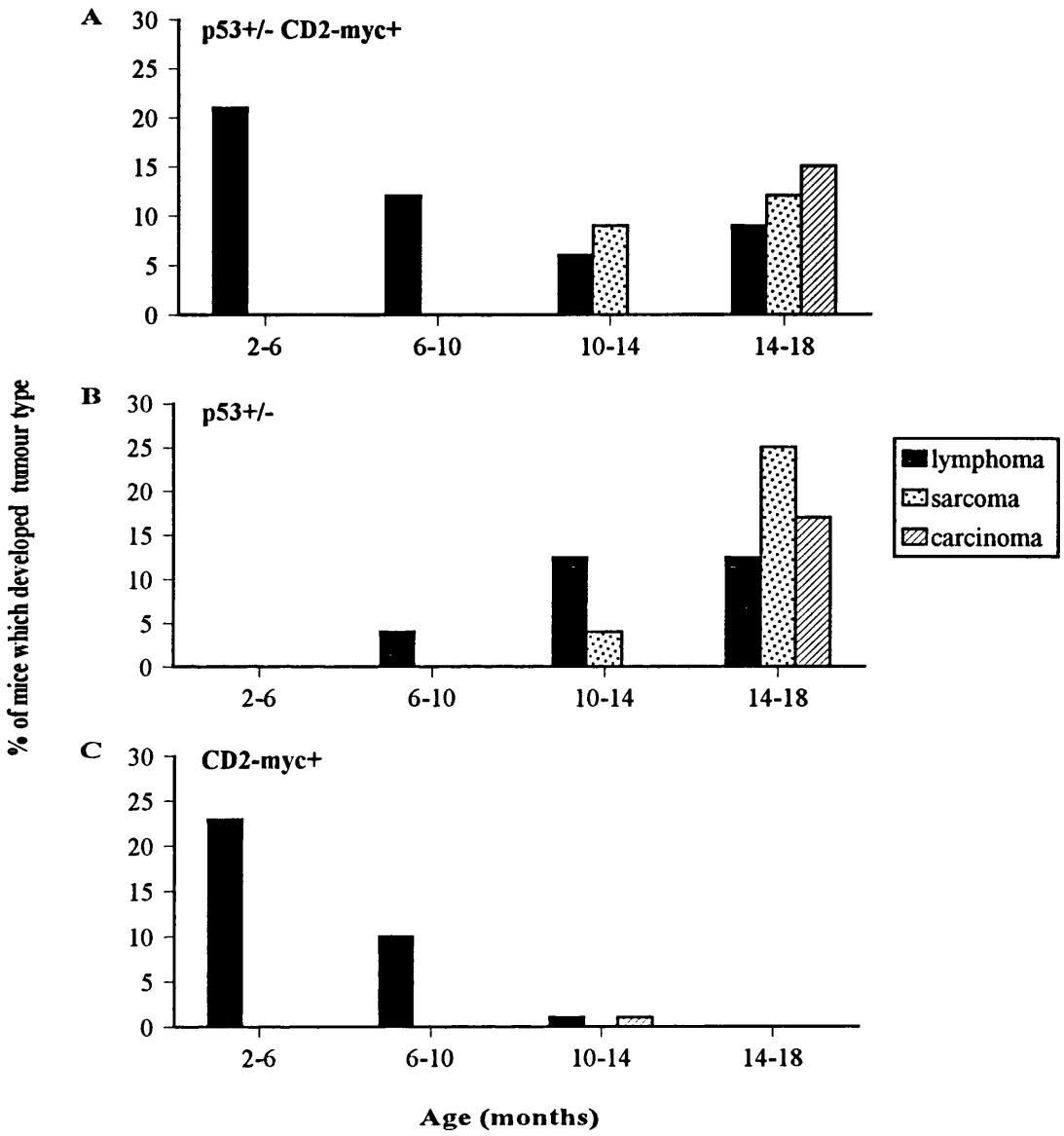

Figure 5.4. Tumour Spectrum of p53+/- CD2-myc+ Mice and Parental Controls.

This figure illustrates the percentage of animals which developed lymphomas, sarcomas and carcinomas at a given time interval. Numbers are expressed as a percentage of the total animal cohort number (n). A) p53+/- CD2-myc+ animals (n=34) are compared against parental groups B) p53+/- (n=24) and C) CD2-myc (n=86). It should be noted that the results displayed in C) represent a combination of the p53+/+ CD2-myc+ mice taken from the current study (n=10) and of an experimental group of CD2-myc+ animals outwith the current cohort (n=76).

5.2.5 CD2-*myc* Transgene Analysis of $p53^{+/-}$/CD2-myc^{+} Tumours.

Evidence from tumour latency and tumour spectrum analysis was consistent with the theory that $p53^{+/-}$/CD2-myc^{+} mice developed additive pathologies associated with either genetic lesion in an independent manner. This hypothesis suggested that tumours developing before nine months of age would be related to the CD2-*myc* transgene while tumours which developed with a later latency would be related to the $p53^{+/-}$ lesion. In order to explore this theory further, $p53^{+/-}$/CD2-myc^{+} tumours were assessed for CD2-*myc* transgene expression. Transgene expression has been invariably associated with neoplastic development in mice which carry the CD2-*myc* transgene and is not detected in the untransformed cells of these mice (Stewart *et al.*, 1993). For the purpose of this study, transgene expression was used as an indication that the CD2-*myc* transgene had contributed to the pathogenesis of $p53^{+/-}$/CD2-myc^{+} tumours. Firstly, the sarcomas and carcinomas, tumour types not normally associated with the CD2-*myc* transgene, which developed in $p53^{+/-}$/CD2-myc^{+} mice were analysed for CD2-*myc* expression. None of the six sarcomas and four carcinomas which were analysed had detectable levels of CD2-*myc* mRNA (Figure 5.5 & Table 5.2). This result reflects the tissue restriction of the CD2-*myc* transgene and validates the hypothesis that these late developing (12 to 18 months), non-lymphoid $p53^{+/-}$/CD2-myc^{+} tumours did not involve the CD2-*myc* lesion.

In contrast to the non-lymphoid tumours, almost all (14/15) of the $p53^{+/-}$/CD2-myc^{+} lymphomas were found to express detectable levels of CD2-*myc* mRNA (Figure 5.6). The only lymphoma which did not express transgene mRNA developed in an aged mouse (18 months) which had a co-existing fibrosarcoma (p/m-46). The latency and pathology exhibited by this mouse were characteristic of $p53^{+/-}$ pathology. All other lymphomas, which developed between 2.5 and 17 months of age were found to express transgene mRNA (Table 5.2). These results suggest that the CD2-*myc* transgene has contributed to the pathogenesis of most of the $p53^{+/-}$/CD2-myc^{+} lymphomas irrespective of the age at which these tumours presented.

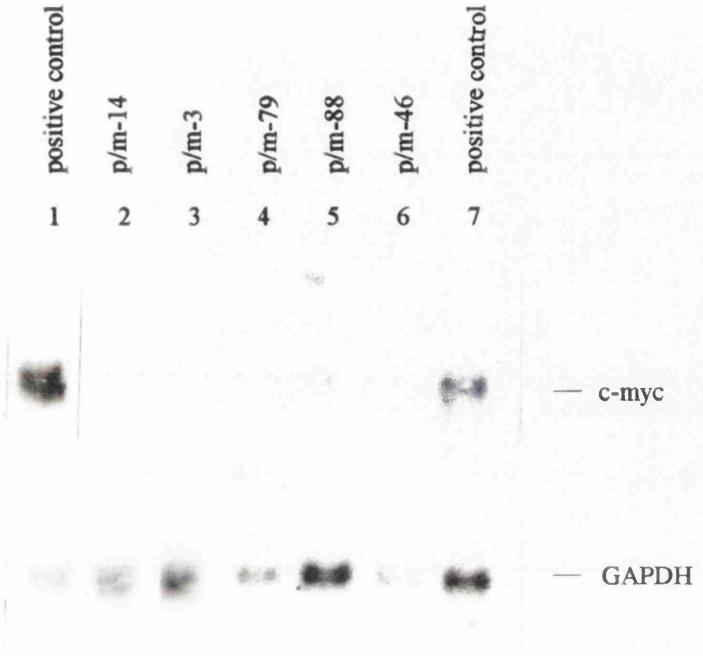

Figure 5.5. Northern Blot Analysis of p53+/- CD2-myc+ Non-lymphoid Tumours.

The sarcomas and carcinomas which developed in p53+/- CD2-myc+ mice were assessed for CD2-myc transgene expression to determine if the transgene had contributed to the pathogenesis of these tumour types. Lanes 1 and 7 are positive controls and lanes 2 to 6 are a representative group of p53+/- CD2-myc+ tumours. The tumour name is given above the lane number. Tumours p/m-14 and p/m-46 were sarcoma type neoplasms and p/m-3, p/m-79 and p/m-88 were carcinoma type neoplasms. Samples were also probed with a GAPDH probe to confirm the presence of RNA in the lanes.

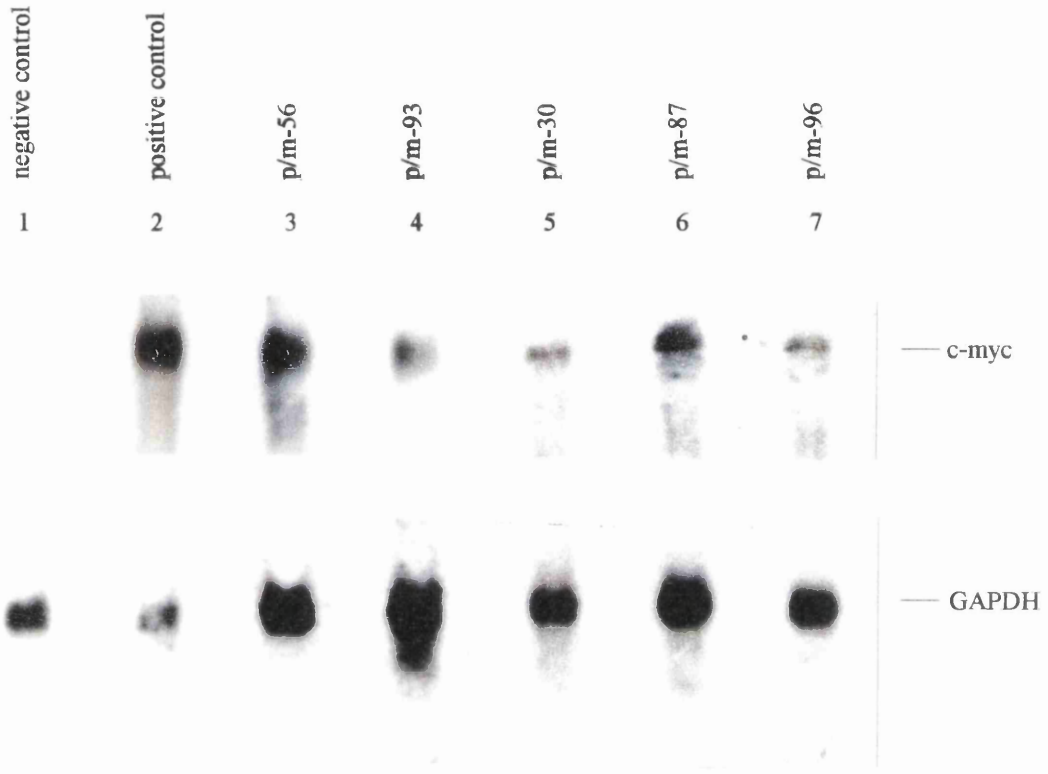

Figure 5.6. Northern Blot Analysis of p53+/- CD2-myc+ Lymphomas.

The thymic lymphomas which developed in p53+/- CD2-myc+ mice were assessed for CD2-myc transgene expression. Lanes 1 and 2 are negative and positive controls respectively. Lanes 3 to 7 represent a selection of p53+/- CD2-myc+ lymphomas with the tumour name given above the lane number. Samples were also probed for the murine GAPDH gene to confirm the presence of RNA in the lanes.

5.2.6 Loss of Heterozygosity Analysis of p53$^{+/-}$/CD2-*myc*$^{+}$ Tumours.

It has been demonstrated previously that tumours which develop in p53$^{+/-}$ mice are frequently associated with loss of the wild type p53 allele (Harvey *et al.*, 1993a; Purdie *et al.*, 1994; Jacks *et al.*, 1994). It was of interest therefore to assess the status of the wild type p53 allele in the p53$^{+/-}$/CD2-*myc*$^{+}$ tumours, especially in the lymphomas of these animals which were positive for transgene expression. It could be postulated that if no loss of the wild type allele was observed in the early p53$^{+/-}$/CD2-*myc*$^{+}$ lymphomas that these tumours were a direct consequence of the CD2-*myc* lesion without the need for p53 loss. Thirteen of the p53$^{+/-}$/CD2-*myc*$^{+}$ lymphomas which developed between 2.5 and 18 months of age were examined by PCR analysis in order to assess loss of heterozygosity. It should be noted that this approach cannot discount the possibility that the wild type allele has been inactivated by mechanisms which are outwith the scope of detection by PCR analysis, for example by point mutation or by MDM2 overexpression. The band representing wild type p53 was absent or obviously under-represented in 12 of the 13 p53$^{+/-}$/CD2-*myc*$^{+}$ lymphomas (Figure 5.7 & Table 5.2). PCR analysis of one lymphoma (p/m-28) showed no significant loss of the wild type p53 band, however Southern blot analysis of this tumour demonstrated that there was a reduction in the intensity of the band representing the wild type allele (Figure 5.8). A selection of six p53$^{+/-}$/CD2-*myc*$^{+}$ sarcomas and carcinomas were also analysed by PCR analysis and found to exhibit reduced intensity of wild type band in most instances (Table 5.2). It was apparent from these results that loss of the wild type p53 allele was occurring in at least some, if not all, of the tumour population in most of the p53$^{+/-}$/CD2-*myc*$^{+}$ tumours. While the remaining hybridisation signals at the wild type band is probably due to contaminating non tumour cells, it is possible that some of the tumours consisted of a mosaic of both p53 positive (p53^{+}) and p53 negative (p53^{-}) populations. PCR analysis also revealed the presence of a rearranged band in a number of the p53$^{+/-}$/CD2-*myc*$^{+}$ lymphomas (Figure 5.7). These rearrangements may result in mutations altering or obliterating the normal p53 function. It is possible however that this band is representative of single stranded DNA, the presence of which could be formally demonstrated either by using a denaturing gel or mung bean nuclease.

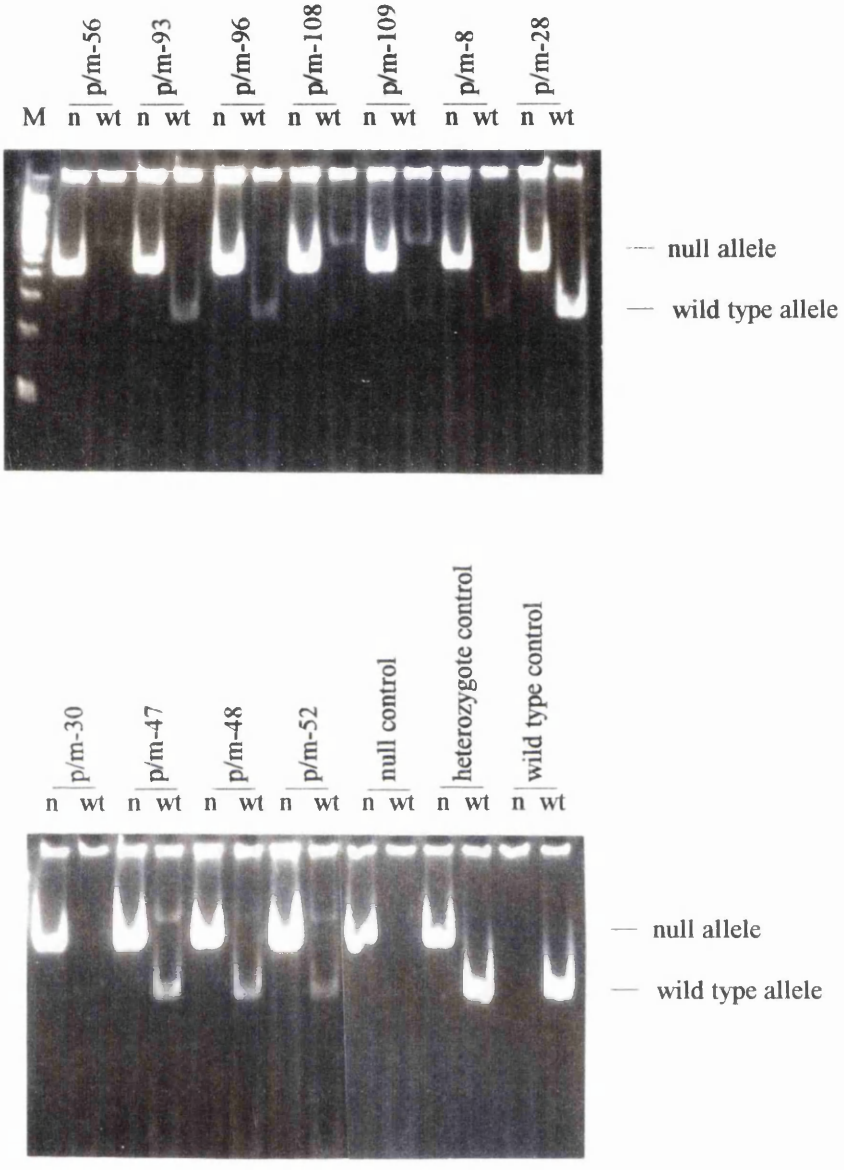

Figure 5.7. PCR Analysis of p53+/- CD2-myc+ Lymphomas.

PCR analysis was used to assess loss of heterozygosity for p53+/- CD2-myc+ lymphomas. Tumour DNA was amplified for both the null allele (n) and the wild type allele (wt). A dramatic reduction in the intensity of the wild type allele can be seen for most of the p53+/- CD2-myc lymphomas. Controls for the null, heterozygote and wild type genotypes are shown. The PCR analysis of these tumour samples and electrophoresis of PCR products was carried out by Mr. C. Johnston.

Table 5.2 A Summary of Analysis of $p53^{+/-}$/CD2-*myc*$^{+}$ Tumours.

Mouse Number	Age (months)	Tumour Type	CD2-*myc* Expression	Loss of Wild Type p53**	Immunophenotype Analysis
p/m-54	2.5	lymphoma	n/d	n/d	n/d
p/m-45	2.5	lymphoma	positive	yes	$CD4^{+}CD8^{+}$ & $CD4^{-}CD8^{-}$
p/m-28	3	lymphoma	positive	yes	$CD4^{-}CD8^{+}$
p/m-93	4.5	lymphoma	positive	yes	$CD4^{+}CD8^{+}$
p/m-109	5	lymphoma	positive	yes	$CD4^{+}CD8^{+}$
p/m-47	5.5	lymphoma	positive	yes	$CD4^{+}CD8^{+}$
p/m-87	6	lymphoma	positive	n/d	n/d
p/m-108	7	lymphoma	positive	yes	$CD4^{+}CD8^{+}$
p/m-48	7.5	lymphoma	positive	yes	$CD4^{+}CD8^{+}$
p/m-77	8	lymphoma	positive	yes	$CD4^{+}CD8^{-}$ & $CD4^{+}CD8^{+}$
p/m-56	10	lymphoma	positive	yes	$CD4^{+}CD8^{+}$
p/m-30	11	lymphoma	positive	yes	$CD4^{+}CD8^{+}$ & $CD4^{-}CD8^{+}$
p/m-96	11	lymphoma	positive	yes	$CD4^{+}CD8^{+}$
p/m-99	12	fibrosarcoma	negative	yes	n/a
p/m-55	12.5	fibrosarcoma	negative	n/d	n/a
p/m-14	13.5	osteosarcoma	negative	yes	n/a
p/m-52	14	lymphoma	positive	yes	$CD4^{+}CD8^{+}$
p/m-79	14.5	adenocarcinoma	negative	n/d	n/a
p/m-25	14.5	spindle cell tumour	negative	n/d	n/a
p/m-88	16	adenocarcinoma	negative	yes	n/a
p/m-59	17	1) adenocarcinoma	n/d	n/d	n/a
		2) osteosarcoma	negative	no	n/a
p/m-3	17	adenocarcinoma	negative	n/d	n/a
p/m-8	17	lymphoma	positive	yes	$CD4^{-}CD8^{-}$
p/m-34	17	osteosarcoma	n/d	n/d	n/a
p/m-46	18	1) lymphoma	negative	yes	$CD4^{+}CD8^{-}$ & $CD4^{-}CD8^{-}$
		2) fibrosarcoma	negative	yes	n/a
p/m-57	18	adenocarcinoma	negative	yes	n/a

n/d = not determined; n/a = not applicable

** loss of wild type p53 was assessed by PCR and Southern blot analysis. The amount of allele loss was highly variable among the tumours ranging from total to partial loss of wild type band (refer to text).

5.2.7 Loss of Wild Type p53 in Metastatic Tissues and Cell Lines.

The finding that some of the $p53^{+/-}$/CD2-*myc*$^{+}$ lymphomas contained both $p53^{+}$ and $p53^{-}$ populations posed the hypothesis that loss of the wild type p53 allele may be occurring as a progression event in CD2-*myc* induced tumours. In support of this hypothesis, loss of the wild type allele was also observed in the metastatic tissues of $p53^{+/-}$/CD2-*myc*$^{+}$ mice which developed lymphomas (Figure 5.8). These non-lymphoid tissues contained levels of wild type p53 comparable to that present in the tumour. Given that these tissues should contain a large population of normal cells, a likely interpretation would be that the metastatic population of infiltrating lymphoid cells consisted mainly of $p53^{-}$ cells.

The *in vitro* growth potential of lymphoma cells isolated from $p53^{+/-}$/CD2-*myc*$^{+}$ mice was investigated by placing these cells in long term cell culture. Out of eleven cell lines which were set up in culture, eight (73%) established in long term culture and three (27%) did not. PCR analysis of five of the established lines demonstrated complete loss of the wild type p53 allele (Figure 5.9). This result suggests that loss of functional p53 activity may be advantageous for the *in vitro* culture of these cell lines. Consistent with this hypothesis, it was previously observed that $p53^{-/-}$/CD2-*myc*$^{+}$ lymphoma cells always established in long term culture while CD2-*myc* cells on a wild type p53 background did not (chapter 3). That total loss of p53 activity is required for *in vitro* growth is in accordance with the role of p53 loss in tumour progression.

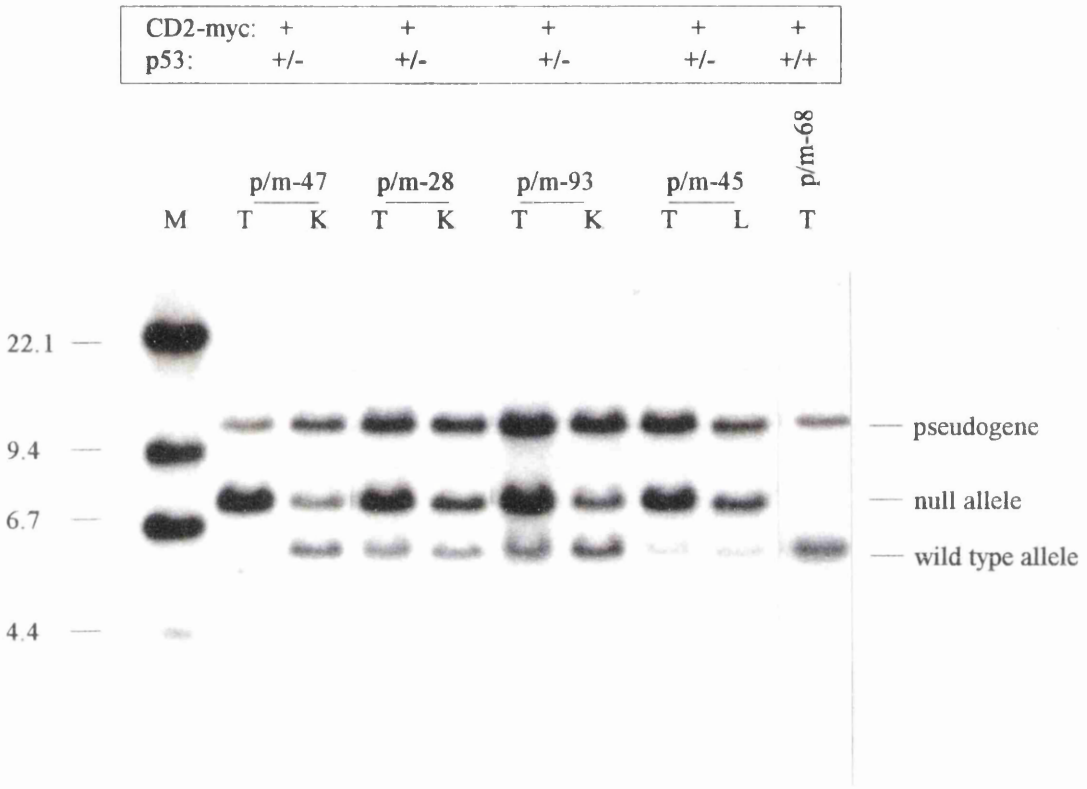

Figure 5.8. Southern Blot Analysis of p53+/- CD2-myc+ Tumours and Metastases.

The status of the wild type p53 allele in the tumours and metastatic tissues of p53+/- CD2-myc+ mice was examined by Southern blot analysis. This figure, which has been adapted from Blyth *et al.*, 1995, illustrates the loss of the wild type p53 allele from the primary tumours (T) and metastatic kidney (K) and liver (L) tissues of p53+/- CD2-myc+ mice. The tumour name and mouse genotype is given for each sample. A control tumour wild type for p53 is also shown (p/m-68). These Southern analyses were carried out by Mrs. A. Terry and have been used here with Anne's kind permission.

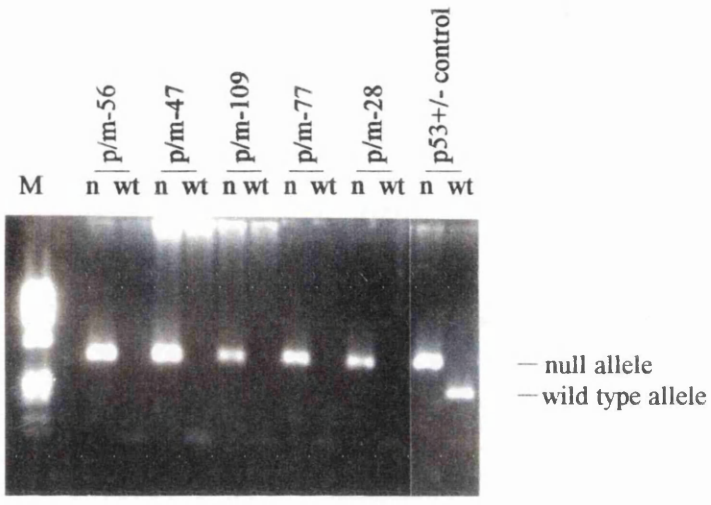

Figure 5.9. PCR Analysis of p53+/- CD2-myc+ Cell Lines.

PCR analysis was used to assess loss of heterozygosity in the cell lines derived from p53+/- CD2-myc+ thymic lymphomas. Each sample was amplified for both the null (n) and wild type (wt) p53 alleles. A p53 heterozygote control is also shown. It can be seen that each of the cell lines has lost the band corresponding to the wild type allele. PCR analysis of the cell lines was carried out by Ms. J. Irvine.

5.3 DISCUSSION.

5.3.1 T-Lymphomagenesis is not Accelerated in Animals Heterozygous for the p53 Null Mutation and the CD2-*myc* Transgene.

Transgenic animals heterozygous for the p53 null mutation ($p53^{+/-}$) and the CD2-*myc* transgene do not accelerate either the CD2-*myc* or $p53^{+/-}$ phenotypes, suggesting that these two events do not collaborate during *in vivo* tumourigenesis. This result contrasts with the dramatic synergy observed between the CD2-*myc* and $p53^{-/-}$ lesions and suggests that the absence of only one wild type p53 allele does not predispose CD2-*myc* transgenic mice to accelerated lymphoma development.

The incidence, latency and spectrum of tumour types which developed in the $p53^{+/-}$/CD2-myc^{+} cohort strongly suggested that these animals died as a result of additive pathologies pertaining to either the CD2-*myc* or $p53^{+/-}$ lesions. A proportion of the $p53^{+/-}$/CD2-myc^{+} mice were found to succumb to tumours before nine months of age. The incidence and latency of these tumours were characteristic of CD2-*myc* transgenic mice. Furthermore, these tumours were all T-cell lymphomas and exhibited the same pathology and immunophenotype as CD2-*myc* tumours which develop in transgenic animals on a wild type p53 background (Stewart *et al.*, 1993). In contrast, $p53^{+/-}$/CD2-myc^{+} tumours which developed after nine months were predominantly non-lymphoid and were found to reflect the tumour pattern normally associated with the $p53^{+/-}$ lesion (Harvey *et al.*, 1993a; Purdie *et al.*, 1994; Jacks *et al.*, 1994). The proportion of mice ultimately succumbing to neoplasia in the $p53^{+/-}$/CD2-myc^{+} cohort was the same as that of the $p53^{+/-}$ control cohort. Therefore the presence of the CD2-*myc* transgene did not alter the overall incidence of tumours in $p53^{+/-}$ mice.

Northern analysis of $p53^{+/-}$/CD2-myc^{+} tumours confirmed that the CD2-*myc* transgene was transcriptionally active in the T-cell lymphomas which developed in $p53^{+/-}$/CD2-myc^{+} mice prior to nine months of age. As would be expected, transgene mRNA was not detected in the non-lymphoid tumours which developed with a latency of more than ten months in $p53^{+/-}$/CD2-myc^{+} mice. This result was consistent with the specificity of the

CD2 element to the T-cell lineage (Greaves *et al.*, 1989) and demonstrated that the CD2-*myc* transgene was not involved in the pathogenesis of $p53^{+/-}$ induced sarcomas and carcinomas. Based on tumour latency analysis (Figure 5.3) it had been expected that lymphomas which developed after ten months of age would, like the sarcomas and carcinomas, be related to the $p53^{+/-}$ lesion and would arise independently of the CD2-*myc* transgene. However four out of five lymphomas occurring between ten and eighteen months were positive for transgene expression (Table 5.2), suggesting that the CD2-*myc* transgene had contributed to the pathogenesis of most of the $p53^{+/-}$/CD2-myc^{+} lymphomas. This result was not in accordance with the hypothesis that $p53^{+/-}$/CD2-myc^{+} mice developed additive pathologies distinct to each of the genetic mutations.

5.3.2 The $p53^{+/-}$ Mutation Co-operates with Eμ-*myc* and MMTV-c-*myc* Transgenes.

The lack of co-operation between the $p53^{+/-}$ and CD2-*myc* lesions in this study is in contrast to two other reports describing dramatic synergy between the $p53^{+/-}$ mutation and c-*myc* transgenes. The Eμ-*myc* and MMTV-c-*myc* transgenes in combination with the $p53^{+/-}$ mutation resulted in accelerated B-cell and T-cell lymphomagenesis respectively (Hsu *et al.*, 1995; Elson *et al.*, 1995). The p53 null mutation was the same for each of these studies discounting this as a possible reason for the differences between these models. It is possible however that differences in genetic background, c-*myc* transgenes and/or tissues types could account for these contrasting results. Although all of these models used the same p53 null mouse (Donehower *et al.*, 1992), the genetic background was distinct in each case, with the $p53^{+/-}$/CD2-myc^{+} animals being an undetermined mix of C57Bl/6, CBA/Ca, 129/Sv and NIH strains. Genetic background has previously been shown to influence the rate and spectrum of tumours in p53 null animals (Harvey *et al.*, 1993b). Nonetheless, it is unlikely that the lack of co-operation between CD2-*myc* and $p53^{+/-}$ is due to genetic background in view of the dramatic synergy observed in $p53^{-/-}$/CD2-myc^{+} littermates (chapters 3 & 4). It is also doubtful that the cell context of this study influences the ability of CD2-*myc* to co-operate with $p53^{+/-}$, particularly as the combination of $p53^{+/-}$ and MMTV-c-*myc* resulted in accelerated T-cell lymphomas (Elson *et al.*, 1995). A more probable explanation for the difference between

the p53$^{+/-}$/CD2-*myc*$^{+}$ model and the other p53$^{+/-}$/c-*myc* models would be with regard to the transgenic constructs. Both the Eμ-*myc* and MMTV-c-*myc* transgenes are constitutively expressed at low levels in various transgenic tissues (Stewart *et al.*, 1984a; Leder *et al.*, 1986; Schmidt *et al.*, 1988). The CD2-*myc* transgene, by contrast, is only found to be expressed in tumourous tissues and is not detected in healthy, untransformed tissue (Stewart *et al.*, 1993). Therefore synergy between c-*myc* transgenes and the p53$^{+/-}$ mutation may require that the transgene is constitutively expressed in transgenic tissues. The presence of overexpressed c-*myc* in these tissues may predispose towards early loss of the second wild type allele, possibly because of increased cell division and the increased chance of accumulating allelic mutations.

5.3.3 p53 Loss as a Progression Event in p53$^{+/-}$/CD2-*myc*$^{+}$ Lymphomas?

Loss of heterozygosity at the p53 locus is a common event in human malignancy (Nigro *et al.*, 1989; Baker *et al.*, 1989; Malkin *et al.*, 1990) and has been recognised to occur as a late progression event in a number of tumour types including colorectal carcinoma (Baker *et al.*, 1990a). Furthermore, loss of the remaining wild type allele frequently occurs in tumours of p53 heterozygote mice (Harvey *et al.*, 1993a; Purdie *et al.*, 1994; Jacks *et al.*, 1994). It is possible that loss of both p53 alleles may also be important for the pathogenesis of tumours in which co-existing p53 and c-*myc* lesions exist. Inactivation of both wild type p53 genes have been observed in human tumour cell lines (Gaidano *et al.*, 1991; Farrell *et al.*, 1991; Inagaki *et al.*, 1994), and in virus-induced mouse erythroleukaemias (Dreyfus *et al.*, 1990) which have a deregulated c-*myc* gene. In addition, Elson *et al.* and Hsu *et al.* reported that loss of the remaining wild type p53 allele frequently occurred in the tumours of their p53$^{+/-}$/c-*myc* models.

Significantly, loss of the wild type p53 allele was exhibited by the CD2-*myc* expressing p53$^{+/-}$/CD2-*myc*$^{+}$ lymphomas. Total allele loss nevertheless was only demonstrated in one of the CD2-*myc* expressing lymphomas. The remaining tumours, while exhibiting partial loss of allele to varying degrees, still retained a proportion of the wild type allele as determined by PCR analysis. It is probable that residual wild type p53 represents contaminating non-tumourous tissue and that functional loss of the wild type allele is

necessary for the initiation of these tumours. However the finding that some of the $p53^{+/-}$/CD2-*myc*$^{+}$ tumours contained apparently substantial populations of wild type p53 may indicate that wild type allele loss was occurring as a late progression event in those tumours. Consistent with this hypothesis, loss of wild type allele was observed in the metastatic tissues and in cell lines derived from the thymic tumours of these mice. The observation that at least partial loss of the wild type allele occurred in $p53^{+/-}$/CD2-*myc*$^{+}$ tumours indicates that there was a selective advantage associated with loss of the second allele. Nevertheless, despite this obvious selective advantage, loss of allele did not influence the overall incidence or latency of $p53^{+/-}$/CD2-*myc*$^{+}$ tumours.

5.3.4 The Role of p53 Mutations in CD2-*myc* Induced Tumours.

It has been demonstrated that the CD2-*myc* transgene dramatically synergises with the $p53^{-/-}$ lesion (chapter 3) but not the $p53^{+/-}$ lesion (this chapter). This observation leads to the suggestion that inactivation of the remaining wild type p53 allele is a rate limiting step in the evolution of the $p53^{+/-}$/CD2-*myc*$^{+}$ lymphomas and demonstrates the importance of the $p53^{-/-}$ lesion as an early event in accelerated CD2-*myc* tumours. The lack of synergy between CD2-*myc* and p53 loss in $p53^{+/-}$/CD2-*myc*$^{+}$ tumours may be a reflection of the time required to lose the second allele which could be superseded by the activation and/or inactivation of other genetic events. Indeed, it has been shown that p53 is not a genetic target in MoMuLV-accelerated CD2-*myc* lymphomas (Stewart *et al.*, 1996) and that other events are involved in the pathogenesis of these tumours.

Evidence from the $p53^{-/-}$/CD2-*myc*$^{+}$ and $p53^{+/-}$/CD2-*myc*$^{+}$ transgenic models described here, and the $p53^{+/-}$/c-*myc* models described by other authors (Hsu *et al.*, 1995; Elson *et al.*, 1995), leads to the conclusion that these mice require at least three (but probably more) genetic events for lymphoma development. One of these events would be the deregulated expression of the c-*myc* oncogene, while inactivation of the p53 tumour suppressor gene would require two additional events. Mice which are genotypically $p53^{+/-}$/Eμ-*myc* and $p53^{+/-}$/MMTV-c-*myc* are born with two of these events. These animals have sustained loss of one p53 allele and constitutively express a c-*myc* transgene in a number of their cells. Loss of the second allele in any one of these cells

will presumably favour tumour development. Inactivation of both p53 alleles in $p53^{-/-}$ /CD2-*myc*$^{+}$ animals means that two genetic events have also occurred in the cells of these mice. Stochastic activation of the CD2-*myc* transgene in one or a few of these cells subsequently provides a third event. By contrast, $p53^{+/-}$/CD2-*myc*$^{+}$ animals are born with only one genetic lesion, namely loss of one wild type p53 allele. Theoretically these mice require activation of the CD2-*myc* transgene and loss of the second p53 allele and/or other unknown events. The fact that a greater number of genetic 'hits' must spontaneously occur in $p53^{+/-}$/CD2-*myc*$^{+}$ tumours reasonably explains the lack of synergy in these mice as compared to $p53^{-/-}$/CD2-*myc*$^{+}$, $p53^{+/-}$/Eμ-*myc* and $p53^{+/-}$/MMTV-c-*myc* mice.

Although the $p53^{+/-}$/CD2-*myc*$^{+}$ model has proven to be a complex one, it is in many ways a more accurate comparison to naturally occurring tumours than the models provided by other authors (Elson *et al.*, 1995; Hsu *et al.*, 1995). It is conceivable that loss of one p53 allele could occur early in the lifetime of a cell but for simultaneous overexpression of an oncogene to occur at this time would be unlikely. Nevertheless, stochastic activation of an oncogene, as seen in the $p53^{+/-}$/CD2-*myc*$^{+}$ model, would not be unexpected. The $p53^{+/-}$/CD2-*myc*$^{+}$ model demonstrates that loss of only one p53 allele would be insufficient to co-operate with the spontaneous activation of the c-*myc* oncogene. It is worth noting however that the p53 null lesion results in deletion of the protein and so requires inactivation of the second allele for loss of function, but p53 mutations which occur in spontaneous tumours may result in dominant negative mutants (Milner *et al.*, 1991) in which case only one p53 allele would need to be mutated for loss of function. It is apparent however that the synergistic interaction between p53 loss and c-*myc* requires inactivation of both wild type p53 alleles. The mechanics of the synergy may be due to abrogation of a c-*myc* induced apoptotic pathway for which loss of both alleles are necessary (Hermeking and Eick, 1994; Wagner *et al.*, 1994). Alternatively, genetic instability generated by total loss of p53 may be necessary for the early stochastic activation of an oncogene, or in this case the CD2-*myc* transgene, which would normally be under the regulation of a p53 cell cycle check-point (Lane, 1992).

CHAPTER 6.

T-CELL LYMPHOMAGENESIS IN P53 NULL MICE INFECTED WITH MOLONEY MURINE LEUKAEMIA VIRUS.

6.1 INTRODUCTION.

Moloney murine leukaemia virus (MoMuLV) is a slow transforming retrovirus which induces murine T-cell lymphomas at a latency of between three and nine months of age (van Lohuizen and Berns, 1990; Tsichlis and Lazo, 1991). MoMuLV induced-lymphomagenesis involves insertional activation of host genes which contribute to the neoplastic process (reviewed by van Lohuizen and Berns, 1990; Kung *et al.*, 1991). Numerous cellular genes which are affected by proviral insertion have been identified (Tsichlis and Lazo, 1991). The most frequently activated genes in MoMuLV-induced T-cell lymphomas are c-*myc*, N-*myc* and *pim*-1 (reviewed by van Lohuizen and Berns, 1990). The common occurrence of these genes suggests that they may be important for the pathogenesis of MoMuLV-induced tumours. It has been demonstrated that MoMuLV infection can accelerate tumourigenesis in several transgenic mice which are predisposed to lymphoma development (Berns *et al.*, 1989; Adams and Cory, 1991a). Proviral insertion of MoMuLV sequences into the host DNA results in activation of cellular genes which collaborate with the transgene in the development of lymphoma. Collaboration of c-*myc* with *pim*-1 and *bmi*-1 has been demonstrated in this way (van Lohuizen *et al.*, 1989; van Lohuizen *et al.*, 1991; Haupt *et al.*, 1991).

The aim of the study described here was to determine if loss of functional p53 could co-operate with MoMuLV infection in the pathogenesis of murine T-cell lymphoma. Mice homozygous for a null allele of the p53 gene are predisposed to the development of T-cell lymphoma (Donehower *et al.*, 1992). Therefore MoMuLV infection of $p53^{-/-}$ mice may provide an opportunity to assess which genes, activated by provirus insertion, act in concert with p53 loss in T-lymphomagenesis. In previous chapters, p53 loss was shown to co-operate with the CD2-*myc* transgene, therefore MoMuLV infection of $p53^{-/-}$/CD2-myc^{+} mice was also carried out to address if additional events to p53 loss and a c-*myc* transgene were required for the development of T-cell lymphoma in this model.

6.2 RESULTS.

6.2.1 Generation of MoMuLV Infected Experimental Animals.

Mice homozygous for the p53 null mutation, $p53^{-/-}$, were generated by crossing together animals which were heterozygous for the null allele, $p53^{+/-}$ (Donehower *et al.*, 1992). Neonatal $p53^{-/-}$, $p53^{+/-}$ and $p53^{+/+}$ animals were infected with MoMuLV within 24 hours of birth and monitored for signs of tumour development over a twelve month period. A small number of infected animals (7/86), of all p53 genotypes, developed thymic atrophy and parasitic infections around the time of weaning. Although the reason for this occurrence is unclear, it is possible that viral inoculation predisposed these animals to immunosuppression (Onions and Jarrett, 1987). In addition, several other young animals died or had to be culled due to unrelated causes, primarily anaesthetic overdose and behavioural problems. These animals were excluded from the long term experimental cohort. Non-infected $p53^{-/-}$ and $p53^{+/-}$ control animals of a comparative genetic background were also monitored for signs of tumour development. The genetic background of all animals was an undetermined mix of C57Bl/6, 129/Sv and NIH strains (Donehower *et al.*, 1992; Kemp *et al.*, 1993).

6.2.2 Accelerated Tumour Development of MoMuLV Infected $p53^{-/-}$ Mice.

The tumour free survival of $p53^{-/-}$ mice infected with MoMuLV is illustrated by Figure 6.1. Survival of infected $p53^{-/-}$ mice was significantly reduced compared to non-infected $p53^{-/-}$ control mice ($p < 0.05$, Mann-Whitney confidence test). MoMuLV infected $p53^{-/-}$ mice were found to succumb to tumour development at a markedly increased rate with 71% (15/21) of infected $p53^{-/-}$ animals dying between the ages of 70 and 150 days of age. By comparison, only 41% (9/22) of non-infected $p53^{-/-}$ mice had developed tumours at this time (Figure 6.1.A). It can be concluded from these observations that MoMuLV accelerated tumour development in $p53^{-/-}$ mice. As shown in Figure 6.1.B, survival of MoMuLV infected $p53^{-/-}$ mice was also significantly reduced compared to MoMuLV infected wild type mice, $p53^{+/+}$ ($p < 0.001$, Mann-Whitney confidence test). Only 10% (2/20) of infected $p53^{+/+}$ mice were found to develop tumours by 150 days of age.

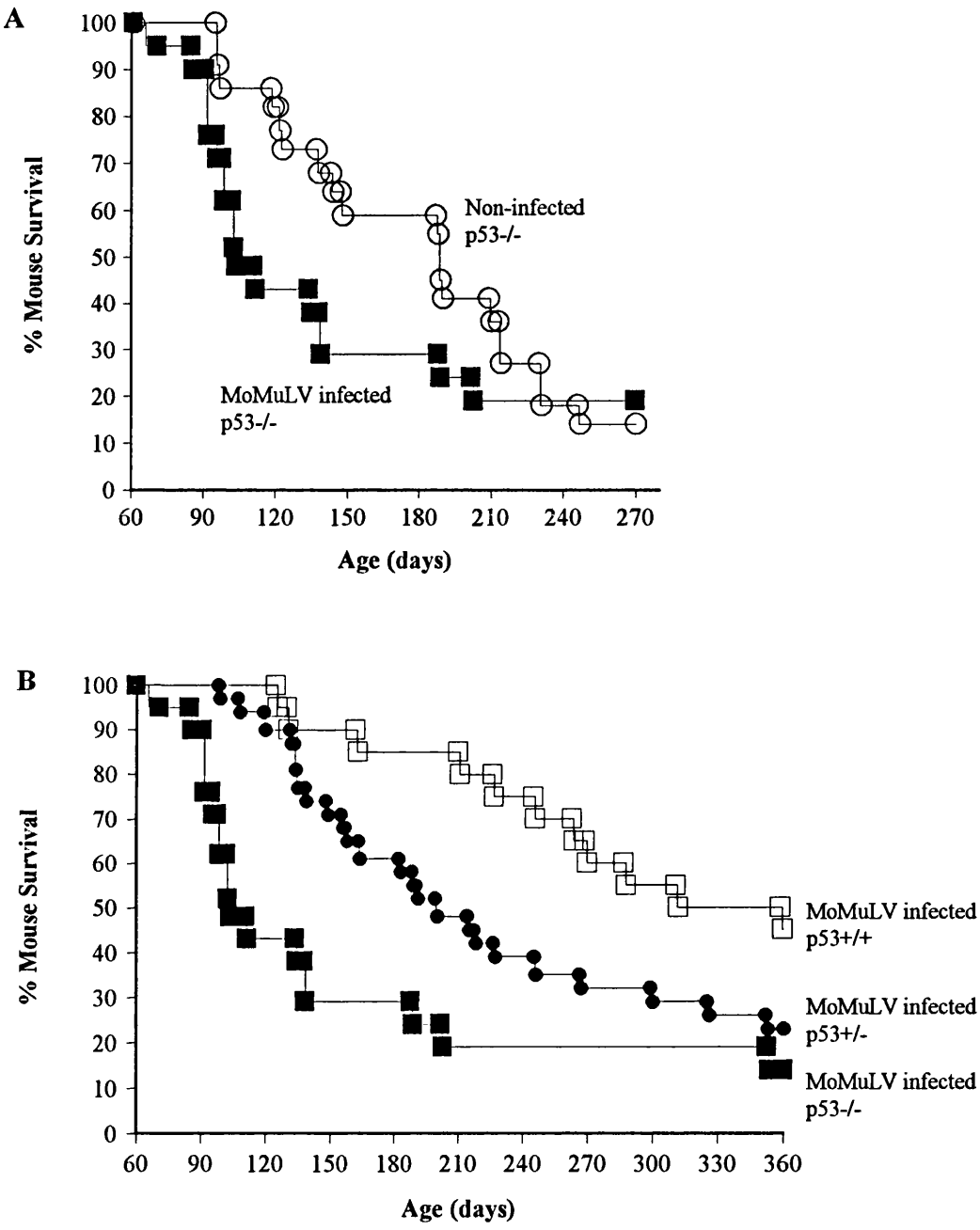

Figure 6.1. Survival Analysis of MoMuLV Infected p53-/- Mice.

A) Compares the percentage of surviving MoMuLV infected p53-/- mice (■, n=21) with non-infected p53-/- mice (O, n=22) at nine months of age. B) Percentage survival of MoMuLV infected p53-/- mice (■, n=21), MoMuLV infected p53+/- mice (●, n=31) and MoMuLV infected p53+/+ mice (□, n=20) at twelve months of age.

An unexpected finding was that a small number of infected $p53^{-/-}$ mice (4/21) survived to beyond 350 days of age, particularly as the majority of infected $p53^{-/-}$ mice had died before 150 days of age. Serum analysis (kindly carried out by Q1 Biotechnology, Glasgow) revealed that these four surviving animals were not viraemic. The absence of a persistent viral infection therefore provides a likely explanation for the extended survival of these animals. While it cannot be ruled out that infection of other animals had been unsuccessful, the presence of viral integrations in the thymic tumours of infected $p53^{-/-}$ mice revealed that MoMuLV had contributed to the pathogenesis of all MoMuLV infected $p53^{-/-}$ lymphomas (Baxter *et al.*, 1996).

6.2.3 Tumour Phenotype of MoMuLV Infected $p53^{-/-}$ Mice.

Previous reports have demonstrated that MoMuLV infected mice develop lymphoblastic T-cell lymphomas involving the thymus, spleen or both organs (Pepersack *et al.*, 1980; Spira *et al.*, 1981; Ott *et al.*, 1992). Although it has been shown that a high number of $p53^{-/-}$ mice also develop T-cell lymphomas (Donehower *et al.*, 1992), a proportion of these animals also develop non-lymphoid neoplasms. It was important therefore to examine the tumour spectrum of MoMuLV infected $p53^{-/-}$ animals to establish if MoMuLV infection had altered the tumour phenotype of $p53^{-/-}$ mice. A comparison of the observed tumour types of infected and non-infected $p53^{-/-}$ groups is given in Table 6.1.

MoMuLV infection of $p53^{-/-}$ mice resulted in an increased proportion of animals developing lymphoma. Gross and histopathological analysis revealed that of 15 infected $p53^{-/-}$ mice for which the cause of death was known, 14 (93%) developed lymphoblastic lymphoma, with 3 mice also presenting with a haemangiosarcoma. Only one infected $p53^{-/-}$ animal, culled at 189 days of age with a giant cell sarcoma, did not have pathological evidence of lymphoma development. It should be noted nevertheless that the viraemic status of this mouse was not investigated and a successful viral infection may not have occurred. The cause of death for two additional animals could not be determined due to extensive autolysis at necropsy. In comparison, of 18 non-infected $p53^{-/-}$ mice for which the cause of death was known, only 13 (72%) were found to

succumb to lymphoma development with 5 of these mice presenting with an additional tumour, usually a haemangiosarcoma. It was apparent therefore that MoMuLV infection had restricted the tumour spectrum of $p53^{-/-}$ mice.

Table 6.1 Tumour Spectrum of $p53^{-/-}$ and MoMuLV Infected $p53^{-/-}$ Mice.

A. Tumour Types of $p53^{-/-}$ Mice.

	Incidence	*Lifespan*
Lymphoma	36% (8/22)	96 - 231 days
Lymphoma & haemangiosarcoma	18% (4/22)	148 - 214 days
Lymphoma & fibrosarcoma	4.5% (1/22)	123 days
Fibrosarcoma	4.5% (1/22)	138 days
Haemangiosarcoma	4.5% (1/22)	231 days
Teratoma & sarcoma	4.5% (1/22)	122 days
Haemangiosarcoma & adenocarcinoma	4.5% (1/22)	188 days
Adenocarcinoma	4.5% (1/22)	435 days
Not determined *	18% (4/22)	n/a

B. Tumour Types of MoMuLV Infected $p53^{-/-}$ Mice.**

	Incidence	*Lifespan*
Lymphoma	65% (11/17)	71 - 135 days
Lymphoma & haemangiosarcoma	17% (3/17)	112 - 139 days
Giant cell sarcoma	6% (1/17)	189 days
Not determined *	12% (2/17)	n/a

* reason for death was not determined by routine pathological investigations, or extensive autolysis prevented diagnosis.

** excludes the four non-viraemic mice which survived beyond 350 days.

Gross pathological examination revealed that the majority of MoMuLV infected $p53^{-/-}$ mice which developed lymphoma displayed marked enlargement of the thymus (9/14).

Frequent, but variable, involvement of the spleen and lymph nodes (8/9) was also observed in these animals. Several other animals displayed a more generalised enlargement of all lymphoid organs (4/14). In these animals the thymus was found to be only slightly enlarged but there was gross enlargement of the spleen, mesenteric and subcutaneous lymph nodes. Metastasis to the kidney was evident in a number of infected $p53^{-/-}$ animals (6/14). One mouse which showed no post-mortem evidence of a lymphoid tumour was found to have a thymic lymphoma when examined by microscopy. Histopathological examination of infected $p53^{-/-}$ tissues was consistent with lymphoblastic lymphoma. Infected $p53^{+/+}$ littermates and infected $p53^{+/-}$ mice were also diagnosed with lymphoblastic lymphoma. There was no difference in the organ distribution of lymphomagenesis between infected $p53^{-/-}$ mice and infected $p53^{+/+}$ or infected $p53^{+/-}$ mice. The pathologies of lymphoma observed in this study were similar to previous reports of MoMuLV infected mice (Pepersack *et al.*, 1980; Spira *et al.*, 1981).

6.2.4 Immunophenotype Analysis of MoMuLV Infected $p53^{-/-}$ Mice.

To investigate the cell type of the lymphomas induced in MoMuLV infected $p53^{-/-}$ mice, cells were isolated from primary tumours, either thymus or lymph node, labelled with monoclonal antibodies (CD3, CD4, CD8, CD45R, αβTCR) and sorted by flow cytometry. All MoMuLV infected $p53^{-/-}$ lymphomas were found to be of T-cell origin as assessed by expression of at least one of the surface markers CD3, CD4, CD8 or αβTCR, or by exclusion of the B-cell marker CD45R. Virtually all of the infected $p53^{-/-}$ lymphomas were positive for, or contained positive populations of $CD3^{+}/\alpha\beta TCR^{+}$ cells but varied in their expression of CD4 and CD8 surface markers (Table 6.2). The tumours of infected $p53^{-/-}$ mice were found to represent a variety of T-cell differentiation stages as based on CD4 and CD8 phenotypes, ranging from immature double-negative to more mature single-positive populations. A similar range of phenotypes was observed in the lymphomas of infected $p53^{+/-}$ and infected $p53^{+/+}$ littermates, and in lymphomas of non-infected $p53^{-/-}$ mice (Table 6.2). Lymphomas of infected $p53^{+/-}$, infected $p53^{+/+}$ and non-infected $p53^{-/-}$ mice were all found to be of T-cell origin. It was noted however that a higher proportion of infected wild type tumours consisted of populations which were negative for expression of CD3 and αβTCR markers.

Table 6.2 Flow Cytometry Analysis of MoMuLV Infected Tumours.

A. CD3 & αβTCR Surface Marker Expression.

Mouse Status		CD3			αβTCR		
p53	MoMuLV	+	+/-	-	+	+/-	-
-/-	+	4/12	8/12	0/12	3/11	7/11	1/11
+/-	+	12/18	4/18	2/18	10/17	4/17	3/17
+/+	+	2/10	4/10	4/10	2/10	3/10	5/10
-/-	-	4/8	2/8	2/8	n/d	n/d	n/d

+ represents a positive population; - represents a negative population and +/- indicates where the tumour had both a positive and negative population. n/d = not determined.

B. CD4 & CD8 Marker Expression.

Mouse Status		Cell Surface Phenotype				
p53	MoMuLV	$CD4^+CD8^-$	$CD4^+CD8^+$	$CD4^-CD8^-$	$CD4^-CD8^+$	mixed*
-/-	+	1/12	4/12	2/12	3/12	2/12
+/-	+	4/18	4/18	0/12	2/18	8/18
+/+	+	0/10	3/10	2/10	2/10	3/10
-/-	-	0/8	3/8	2/8	0/8	3/8

* mixed populations consisted of at least more than one major T-cell population, often with a diffuse and heterogeneous staining pattern.

The phenotypic heterogeneity of MoMuLV T-cell lymphomas, irrespective of p53 genotype, is consistent with previous reports of MoMuLV tumours (Lazo *et al.*, 1990; Ott *et al.*, 1992). The $p53^{-/-}$ mutation therefore did not obviously restrict the T-cell phenotype of MoMuLV lymphomas. A number of individual tumours, of all p53 groups, were found to exhibit a heterogeneous T-cell phenotype with more than one major T-cell population. Diversity within individual MoMuLV infected tumours has also been reported by Lazo *et al.* (1990). These authors suggested that the phenotypic

heterogeneity of MoMuLV-induced T-cell tumours may be due to viral transformation of immature T lymphocytes which undergo limited differentiation during oncogenesis.

6.2.5 Tumour Development of Heterozygote Mice infected with MoMuLV.

MoMuLV infected heterozygote mice, $p53^{+/-}$, were found to succumb to tumour development at a rate significantly faster than infected wild type controls ($p < 0.05$, Mann-Whitney confidence test) but displayed intermediate survival compared to infected $p53^{-/-}$ animals (Figure 6.1.B). This observation suggests that loss of one p53 allele may predispose to accelerated tumour development upon MoMuLV infection. Infected $p53^{+/-}$ animals also developed tumours with a much reduced latency when compared to non-infected $p53^{+/-}$ animals (Figure 6.2). Consistent with results of Harvey *et al.* (1993a), non-infected p53 heterozygotes spontaneously developed a wide range of tumour types with a latency greater than seven months (Table 6.3 & Figure 6.2). By contrast, tumour development in MoMuLV infected heterozygotes occurred from three months of age and was almost exclusively restricted to lymphoma development (Table 6.3). Only two infected $p53^{+/-}$ animals, sacrificed after 300 days of age, failed to develop lymphoma. It was apparent therefore that MoMuLV infection had skewed the tumour phenotype of $p53^{+/-}$ animals.

Table 6.3 Tumour Spectrum of Infected and Non-infected $p53^{+/-}$ Mice.

Tumour Type	MoMuLV Infected $p53^{+/-}$ Mice		Non-infected $p53^{+/-}$ Mice	
	Incidence	*Lifespan*	*Incidence*	*Lifespan*
Lymphoma	71% (22/31)	99 - 300 days	3% (1/38)	502 days
Sarcoma	6% (2/31)	326 - 353 days	53% (20/38)	216 - 601 day
Carcinoma	0	n/a	16% (6/38)	357 - 554 days
Not determined*	0	n/a	18% (7/38)	n/a

* cause of death was not determined during routine pathological examinations, or extensive autolysis had occurred. n/a = not applicable.

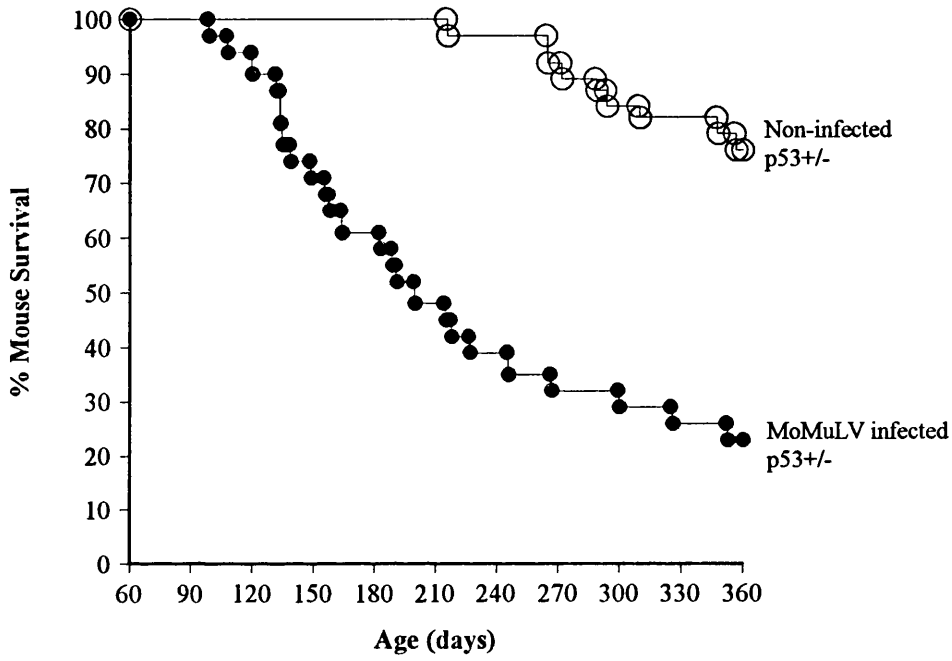

Figure 6.2. Reduced Survival of p53+/- Mice Infected with MoMuLV.

This figure compares the survival of MoMuLV infected p53+/- mice (●, n = 31) with non-infected p53+/- mice (O, n = 38) over a twelve month observation period.

Gross pathological examination of infected $p53^{+/-}$ lymphomas revealed gross thymic enlargement with variable metastasis in 16/22 (73%) of animals and disseminated lymphomas with gross enlargement of the spleen and lymph nodes in 6/22 (27%) of mice. Thirteen of the infected $p53^{+/-}$ animals also had metastasis to non-lymphoid tissues, namely kidney or liver. As discussed previously, all MoMuLV infected $p53^{+/-}$ lymphomas were of T-cell origin (Table 6.2) and showed phenotypic heterogeneity as commonly observed with MoMuLV infected tumours. The status of the wild type p53 allele was examined in six of the MoMuLV infected $p53^{+/-}$ thymic lymphomas and was found to be at least partially lost as assessed by reduced intensity of the wild type band by Southern analysis (E. Baxter, personal communication; Baxter *et al.*, 1996). Loss of the wild type allele was also observed in the metastatic tissues of $p53^{+/-}$ mice indicating that loss was indeed associated with tumour progression.

6.2.6 Cell Line Establishment.

A number of thymic tumours derived from MoMuLV infected $p53^{-/-}$, $p53^{+/-}$ and $p53^{+/+}$ animals were examined for their *in vitro* growth capacity. Successful cell line establishment was achieved for 9/10 of the infected $p53^{-/-}$ lymphomas, 3/12 of the infected $p53^{+/-}$ lymphomas and 3/8 of the infected $p53^{+/+}$ tumours. All of the MoMuLV infected $p53^{+/-}$ lymphomas which established in long term culture were found to have completely lost the wild type p53 allele (Baxter *et al.*, 1996). The primary tumours of these three lines had also shown loss of allele.

6.2.7 MoMuLV Infection of $p53^{-/-}$/CD2-*myc*$^{+}$ Mice.

It was demonstrated in chapters 3 and 4 that the combination of the $p53^{-/-}$ mutation with the CD2-*myc* transgene resulted in accelerated tumourigenesis but that additional events were required. MoMuLV infection can accelerate tumourigenesis in both parental CD2-*myc* and $p53^{-/-}$ animals (Stewart *et al.*, 1993; this chapter), therefore it was of interest to determine if MoMuLV could accelerate tumourigenesis in $p53^{-/-}$/CD2-*myc*$^{+}$ transgenic mice. To address this, CD2-*myc* and $p53^{-/-}$ parental controls (Stewart *et al.*, 1993; Donehower *et al.*, 1992) were crossed to generate $p53^{+/-}$/CD2-*myc*$^{+}$ mice. First

generation $p53^{+/-}$/CD2-*myc*$^{+}$ animals were subsequently bred together to create a cohort of $p53^{-/-}$/CD2-*myc*$^{+}$ mice (n = 11) which were inoculated with MoMuLV within 24 hours of birth. Non-infected $p53^{-/-}$/CD2-*myc*$^{+}$ controls (n = 18), described previously (chapter 3), were of comparable genetic background consisting of C57Bl/6, CBA/Ca, 129/Sv and NIH strains.

MoMuLV infection of $p53^{-/-}$/CD2-*myc*$^{+}$ mice significantly accelerated tumourigenesis as compared to non-infected $p53^{-/-}$/CD2-*myc*$^{+}$ mice ($p < 0.001$, Mann-Whitney confidence test). Infected $p53^{-/-}$/CD2-*myc*$^{+}$ mice died with a latency of between 41 and 63 days of age compared to that of between 60 and 86 days for non-infected mice (Figure 6.3). All MoMuLV infected $p53^{-/-}$/CD2-*myc*$^{+}$ mice developed thymic lymphoma with gross enlargement of the thymus and consistent metastatic spread to the spleen, mesenteric lymph nodes and subcutaneous lymph nodes. Virtually all animals (10/11) also displayed pathological evidence of metastasis to the kidney. It can be concluded therefore that MoMuLV, CD2-*myc* and $p53^{-/-}$ collaborate to induce accelerated lymphomagenesis in this transgenic mouse model. The latency of at least 41 days however suggests that further events may still be required. This hypothesis is supported by the observation that MoMuLV/$p53^{-/-}$/CD2-*myc*$^{+}$ lymphomas are not polyclonal but consist of clonal populations as assessed by TcR and IgH gene rearrangements (E. Baxter, personal communication).

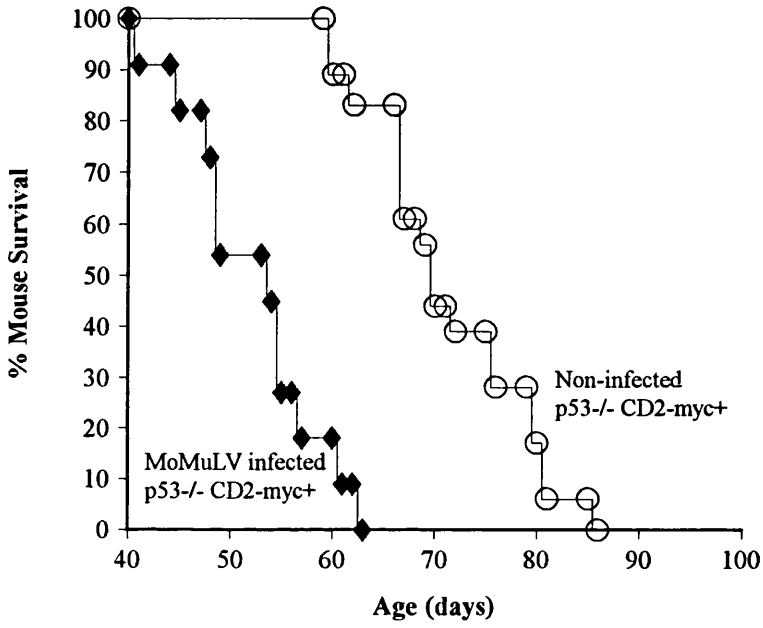

Figure 6.3. Tumour Free Survival Analysis of p53-/- CD2-myc+ Mice Infected with MoMuLV.

The tumour free survival time of MoMuLV infected p53-/- CD2-myc+ mice (◆, n=11) was dramatically reduced compared to non-infected p53-/- CD2-myc+ mice (O, n=18). All infected and non-infected p53-/- CD2-myc+ mice developed thymic lymphoma.

6.3 DISCUSSION.

6.3.1 Accelerated T-Lymphomagenesis in MoMuLV Infected $p53^{-/-}$ Mice.

Viral infection of homozygous p53 null mice has revealed that MoMuLV can co-operate with functional inactivation of the p53 gene to accelerate the onset of murine T-cell lymphomas. MoMuLV infected $p53^{-/-}$ mice were found to succumb to tumour development faster than infected wild type littermates and non-infected $p53^{-/-}$ control mice. The intermediate survival of MoMuLV infected heterozygote mice and the loss of the wild type allele from the tumours of these mice further supports the hypothesis that loss of p53 function may be important for the pathogenesis of MoMuLV induced tumours.

Inactivation of the p53 gene has also been described in the progression of virally transformed leukaemias induced by Friend murine leukaemia virus (F-MuLV) and Abelson murine leukaemia virus (Wolf and Rotter, 1984; Mowat *et al.*, 1985; Chow *et al.*, 1987; Ben-David *et al.*, 1988; Munroe *et al.*, 1990). Loss of p53 function, often by gene rearrangement, appears to be an obligatory event for the pathogenesis of F-MuLV induced mouse erythroleukaemias (Ben-David and Bernstein, 1991). The importance of p53 loss in the evolution of F-MuLV was supported by the infection of p53 transgenic mice with F-MuLV. Transgenic mice which express a dominant-negative mutated p53 allele (Lavigueur *et al.*, 1989) developed accelerated onset of late stage erythroleukaemia compared to infected wild type control animals (Lavigueur and Bernstein, 1991).

6.3.2 Weak Synergy Between MoMuLV and p53 Loss.

The synergy between the $p53^{-/-}$ mutation and MoMuLV infection was however not as pronounced as the synergy observed with each of these lesions in concert with the CD2-*myc* transgene. Transgenic mice co-expressing the CD2-*myc* transgene and the $p53^{-/-}$ mutation succumbed to rapid T-cell lymphoma development before 86 days of age (chapter 3). Similarly, all MoMuLV infected CD2-*myc* transgenic mice developed tumours between 53 and 84 days of age (Stewart *et al.*, 1993). The weaker synergy

observed between MoMuLV and the $p53^{-/-}$ mutation could be due to differences in the genetic background of infected animals as susceptibility to MoMuLV infection can be determined by mouse strains (Tsichlis and Lazo, 1991). It is also possible that viral inoculation of some $p53^{-/-}$ mice did not result in established infection. Indeed four animals which were found to survive beyond ten months of age were examined and found not to be viraemic. However viral infection was successful in most animals as evident from the unique proviral integrations in the thymic tumours of infected $p53^{-/-}$ mice (Baxter *et al.*, 1996). Although non-persistent viral infection of a few animals and genetic background could play a small part in determining the survival of MoMuLV infected $p53^{-/-}$ mice, it is unlikely that these alone could account for the relatively weak synergy between $p53^{-/-}$ and MoMuLV.

It could be proposed that the weaker synergy of MoMuLV with $p53^{-/-}$ may reflect an overlap in the functions of p53 loss and MoMuLV infection in the pathogenesis of lymphoma development. Likewise, it was reported that the lack of synergy between the $p53^{-/-}$ mutation and a *bcl*-2 transgene was due to a functional overlap of these genetic events (Marin *et al.*, 1994). It was suggested by Marin and co-workers that p53 loss and overexpression of the *bcl*-2 oncogene were effectors of a common apoptotic pathway, both acting to inhibit cell death. It is possible that MoMuLV infection may, like *bcl*-2, have anti-apoptotic properties. This would explain the lack of synergy of MoMuLV infected *bcl*-2 transgenic mice described by Acton *et al.* (1992) and the weak synergy described by other workers (Shinto *et al.*, 1995). However acceleration of tumourigenesis in MoMuLV infected $p53^{-/-}$ mice, and *bcl*-2 transgenic mice, does indicate that such a functional overlap cannot be completely redundant and that MoMuLV may also result in activation of other oncogenic events which co-operate with loss of p53 and overexpression of *bcl*-2.

Nevertheless, the results of MoMuLV infected $p53^{-/-}$ and *bcl*-2 mice suggests that the collaboration between MoMuLV infection and survival genes may be less potent than the collaboration between MoMuLV and proliferating genes. For example, MoMuLV strongly collaborates with c-*myc* and *pim*-1 transgenes to accelerate lymphomagenesis (Stewart *et al.*, 1993; van Lohuizen *et al.*, 1989). As MoMuLV replication requires

proliferating cells, the potent collaboration of MoMuLV with c-*myc* and *pim*-1 may be due to the enhanced frequency of viral insertion thereby increasing the chance of gene activation or inactivation. Alternatively, a larger set of virus-activated oncogenes may collaborate with c-*myc* and *pim*-1 whereas a specific subset of less potent oncogenes will collaborate with p53 loss or *bcl*-2. Furthermore, if MoMuLV has anti-apoptotic activities then an enhanced cell survival signal will effectively synergise with the proliferation signal of an oncogene in a similar manner to the synergy observed between *bcl*-2 with c-*myc* (Strasser *et al.*, 1990) and $p53^{-/-}$ with c-*myc* (chapter 3; Blyth *et al.*, 1995).

6.3.3 Gene Targets of Proviral Insertion.

MoMuLV induces oncogenic effects by proviral insertional mutagenesis (van Lohuizen and Berns, 1990). Characterisation of the sites of MoMuLV provirus integration has previously led to the identification of numerous collaborating oncogenes in lymphomagenesis (van Lohuizen *et al.*, 1989; van Lohuizen *et al.*, 1991; Haupt *et al.*, 1991). MoMuLV infection of $p53^{-/-}$ mice provided an opportunity to investigate which genetic activation events preferentially co-operated with the loss of p53. In a study carried out by Dr. Euan Baxter, 3/11 $p53^{-/-}$ tumours were found to have rearrangements at the c-*myc* gene (Baxter *et al.*, 1996). This result is consistent with the observed synergy between the $p53^{-/-}$ mutation and the CD2-*myc* transgene (chapter 3) confirming the strong synergistic relationship between these genetic events in T-cell lymphoma development. No rearrangements at *bmi*-1, *ahi*-1, N-*myc* or *pim*-1 were observed in infected $p53^{-/-}$ mice but one tumour did have an insertion at *pal*-1 (Baxter *et al.*, 1996). Although *pim*-1 insertions were not found in $p53^{-/-}$ tumours, they occurred in $p53^{+/-}$ tumours which had lost the wild type allele suggesting that *pim*-1, like c-*myc*, was a potential collaborating event in co-operation with p53 loss. Insertions at c-*myc* and *pim*-1 were also demonstrated in the tumours of MoMuLV infected *bcl*-2 transgenic mice (Shinto *et al.*, 1995).

Retroviral insertion of F-MuLV sequences has been shown to occur within the p53 gene leading to functional inactivation (Chow *et al.*, 1987; Ben-David *et al.*, 1988). Indeed

one cell line was described in which F-MuLV sequences had integrated independently into both alleles of the p53 gene (Hicks and Mowat, 1988). In contrast to F-MuLV, MoMuLV apparently does not integrate within the p53 gene as assessed by examination of six MoMuLV infected $p53^{+/-}$ tumours (Baxter *et al.*, 1996). The lack of viral insertion of MoMuLV sequences in the p53 gene is consistent with the hypothesis that p53 inactivation and MoMuLV infection share functional properties. However that loss of the wild type p53 allele was found to occur in $p53^{+/-}$ tumours suggests that inactivation of p53 function, evidently by a non-viral mechanism, can contribute to MoMuLV induced lymphomagenesis.

6.3.4 MoMuLV Determines the Phenotype of Infected $p53^{-/-}$ Tumours.

Gross pathology and flow cytometry analysis of MoMuLV infected $p53^{-/-}$ tumours revealed that almost all mice developed T-cell lymphoma, although three animals also had co-existing haemangiosarcomas. As a higher proportion of infected mice developed T-cell lymphoma compared to non-infected mice it is likely that MoMuLV infection restricted the tumour phenotype of $p53^{-/-}$ mice. Likewise, MoMuLV infection was found to skew the tumour phenotype of $p53^{+/-}$ mice. Flow cytometry analysis of MoMuLV $p53^{-/-}$ infected lymphomas indicated phenotypic heterogeneity with tumours representing a variety of T-cell differentiation stages. Heterogeneous T-cell phenotypes, which occurred between and within individual tumours, were also found in infected $p53^{+/-}$ and $p53^{+/+}$ groups. These results are consistent with other studies which described phenotypic heterogeneity of MoMuLV infected tumours (Lazo *et al.*, 1990; Ott *et al.*, 1992). It was apparent therefore that MoMuLV was the phenotypic determinant of MoMuLV infected $p53^{-/-}$ tumours. It could be proposed that initiation of infected $p53^{-/-}$ tumours was a result of MoMuLV and that loss of functional p53 contributed to tumour growth by reducing the time for tumour progression. In support of this hypothesis clonal integrations of viral sequences were observed in the infected $p53^{-/-}$ tumours indicating that viral insertion had preceded the expansion of the tumour clone (E. Baxter, personal communication).

6.3.5 Collaboration Between MoMuLV, the CD2-*myc* Transgene and Loss of p53 In T-cell Lymphomagenesis.

The combined synergistic action of p53 loss with the CD2-*myc* transgene has been detailed in previous chapters. However, the clonality and latency of tumours in $p53^{-/-}$ /CD2-myc^{+} mice suggested that these two events alone were insufficient for lymphoma development. This interpretation has been confirmed by infection of $p53^{-/-}$/CD2-myc^{+} mice with MoMuLV which resulted in a further acceleration of tumour onset. All infected mice developed thymic lymphoma similar to uninfected $p53^{-/-}$/CD2-myc^{+} mice but at a much reduced latency. MoMuLV infection, which has previously been shown to collaborate with the CD2-*myc* transgene (Stewart *et al.*, 1993) and the $p53^{-/-}$ mutation (this chapter), can therefore act in co-operation with these two events in T-cell lymphomagenesis. However as infected $p53^{-/-}$/CD2-myc^{+} mice developed clonal tumours after a latency of 40 days it is likely that more than one additional event was required for tumour development. Exploitation of provirus insertional mutagenesis of infected $p53^{-/-}$ /CD2-myc^{+} tumours should narrow the search for those genes which can preferentially collaborate with c-*myc* deregulation and p53 loss. It is possible that this approach will identify a set of genes not previously considered to be strong oncogenes but whose function is distinct from the effects of c-*myc* deregulation and p53 inactivation and specifically complements these actions in the pathogenesis of murine T-cell lymphomas.

FUTURE WORK

The results described here demonstrate that certain genetic lesions can collaborate in the pathogenesis of murine T-cell lymphoma. It has been shown that inactivation of the p53 tumour suppressor gene can co-operate with a human c-*myc* transgene to dramatically reduce the latency time to lymphoma development but that loss of both functional p53 alleles is required for this synergy. These two events alone are insufficient for tumourigenesis however and it is likely that other events, which can be provided by MoMuLV infection, are required for tumour development.

Future work will concentrate on exploiting the $p53^{-/-}$/CD2-myc^{+} transgenic mouse model to identify those genes which can co-operate with c-*myc* and p53 loss in T-cell lymphomagenesis, and conversely, which oncogenic events are rendered redundant by the presence of deregulated c-*myc* and the absence of p53. These studies may allow the assigning of oncogenes to complementation groups based on their ability to co-operate. The identification of collaborating genes in the $p53^{-/-}$/CD2-myc^{+} model may reveal distinct mechanisms which specifically complement the effects of c-*myc* and p53 loss. Studies of proviral insertional mutagenesis, which are currently underway, have not yet identified any known common integration sites in the $p53^{-/-}$/CD2-myc^{+} tumours. It is possible therefore that these studies may uncover novel cancer associated genes. These genes may not necessarily be involved in the initiation of tumour development but may influence secondary tumour characteristics such as malignant progression and metastasis.

The mechanism of how the CD2-*myc* transgene collaborates with p53 loss to induce T-cell lymphomagenesis remains unclear. It will be important to investigate this further to understand the nature of collaboration between c-*myc* and p53 loss in tumourigenesis. The primary effects of deregulated c-*myc* on thymocytes, whether cell proliferation or cell death, have been difficult to determine using the current CD2-*myc* mouse model. To address this issue, a transgenic model in which c-*myc* expression can be induced in thymocytes is currently being developed. Such a model may delineate the cellular environment and genetic events that can influence the fate of c-*myc* expressing thymocytes.

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