The individual and co-operative effects of oncogenes *myb* and *kit* in murine haemopoietic cells.

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ABSTRACT

The oncogenes *myb and kit* that respectively encode a nuclear transcription factor and a tyrosine kinase cell surface receptor, have both been implicated in human and murine malignancies. Enforced-expression of various forms of Myb and Kit in primary murine haemopoietic cells was investigated to determine the functions of these two oncoproteins separately and in synergy *in vitro*. The effect of an activated C-terminally truncated form of Myb was also examined *in vivo*.

To determine if structural alteration was necessay for transformation by Myb, over-expression of WTMyb in murine foetal liver cells was compared to CT3Myb (a C-terminally truncated oncogenic form of c-Myb), by an *in vitro* clonogenic assay and growth of cell lines in culture. Haemopoietic cells transformed by WTMyb were of the early myeloid lineage and dependent on exogenous growth factor, similar to foetal liver cells transformed by CT3Myb. In both cases, increase in cell density during culture led to an increase in colony formation, but CT3Myb infected cells were able to form the maximal number of colonies at a much lower cell density than that of WTMyb infected cells. Therefore, WTMyb was able to transform haemopoietic cells *in vitro*, but structural alteration of Myb (as in CT3Myb), enhanced transformation and resulted in a more complete block in differentiation.

To determine the effects of over-expression of Myb *in vivo*, a murine bone marrow transplantation system of repopulating mice with CT3*myb*-infected bone marrow compared to control vector (Neo) infected bone marrow was established. Retroviral *myb* was detected by PCR in the peripheral blood, spleen and bone marrow of some mice repopulated with *myb* infected bone marrow, and retroviral Myb was also found to be expressed in the spleen of some of these mice. However, white cell counts, blood smears, analysis of bone marrow and spleen cells did not exhibit any differences when compared to those of control mice and there was no difference in or the number of colonies formed by bone marrow cells plated in GM-CSF. These data as well as results from other studies suggest that continuous expression of activated Myb is insufficient to cause any haemopoietic malignancy and that subsequent

mutations in other genes or 'secondary steps' are required for complete transformation, in which MTHC are factor-independent.

A mutation in c-Kit (V816), identified in a human mast cell line and also found in patients with mastocytosis, causes constitutive activation of Kit and confers factor-idependence in myelomonocytic FDC-P1 and mast IC-2 cell lines. Primary foetal liver cells were infected with retroviruses carrying V816kit, WTkit or vector alone and analysed *in vitro*. There was a dramatic increase in megakaryocytes in V816Kit infected cells compared to control cells after 1 week in culture in the absence of added growth factors. In the presence of ligand (SCF), V816Kit and WTKit expressing populations gave rise to increased numbers of megakayocytes as well a few mast cells. V816Kit expressing populations failed to give rise to long term factor-independent cell lines or colonies. However, in combination with activated Myb, V816Kit was able to give rise to proliferating cells of the myeloid lineage that exhibit mature characteristics.

To observe the co-operative effects of Myb and Kit, V816Kit and WTKit were introduced into an established MTHC line maintained in GM-CSF. The V816Kit expressing cells, able to grow in culture without exogenous growth factor, showed greater survival and proliferation rates than the parent cells, and were tumourigenic in syngeneic mice. WTKit expressing cells could be maintained in the presence of human SCF alone but proliferated at lower levels and exhibited decreased clonogenic potential compared to parental cells. The introduction of Kit also induced phenotypic and morphological changes in the total cell populations, especially increased numbers of adherent differentiated cells. Additionally, large to giant sized cells expressing esterases and surface markers of both macrophage and neutrophil lineages, and displaying phagocytic potential, were observed in these adherent populations *in vitro*. These cells showed morphological features similar to that of histiocytes. V816Kit expressing cells were highly tumourigenic with tumours being heterogeneous containing mature cells as well as those resembling histiocytes.

Our results overall indicate that activated Myb induces immortalization of myeloid cells and activated Kit confers factor-independence and increased survival, thereby co-

operatively inducing complete transformation. The mixed populations derived by over-expressing both oncogenes in haemopoietic cells, resemble the cells from patients of acute myeloid leukaemia - with histiocytic differentiation (AML-M5c) that has recently been documented. Myb and Kit, or other closely related onco-proteins, may be relevant in these newly identified types of leukaemia.

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference has been made in the text. I give consent for this thesis to be made available for loan and photocopying.

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Oncogenic Myb and Oncogenic Kit induce proliferation, differentiation, activation and tumourigenicity of myeloid cells.

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Oncogenic Transformation by c-Kit.

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ABBREVIATIONS

Aa	amino acid
Ab-ML	Ab-MLV induced myeloid leukaemia
Ac	Acetate
AML	acute myeloid leukaemia
AP	Alkaline Phosphatase
APAAP	Alkaline phosphatase anti-alkaline phosphatase
Az	Azide
A_{260}	Absorbance (at 260nm)
BFU	Burst forming unit
BM	bone marrow
BMMC	bone marrow derived mast cells
bp	base pairs
BSA	bovine serum albumin
C-terminus	carbxyl-terminus
CBP	CREB binding protein
cDNA	complememtary DNA
CFU-S	spleen-colony forming unit
CIP	calf intestinal phosphatase
CML	Chronic myeloid Leukaemia
cpm	counts per minute
CSF-1	colony stimulating factor-1
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DTT	1,4-Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
Epo	Erythropoietin
FCS	foetal calf serum
FITC	Fluorescein-isothiocyanate
FLC	Foetal liver cells
5FU	5-Fluorouracil

G-CSF	granulocyte colony stimulating factor
G418	Geneticin
GM	granulocyte / macrophage
GM-CSF	granulocyte-macrophage colony stimulating factor
HBBS	Hank's balanced salt solution
HCl	hydrochloric acid
HCP	Haemopoietic Cell Phosphatase
HEPES	N-2-Hydroxyethylpiperazine N'2-etane sulphonic acid
HRP	Horse-radish Peroxidase
HTH	helix-turn-helix
hu	human
Ig	immunoglobulin
IL (e.g. IL-3)	Interleukin (e.g. Interleukin-3)
IMDM	Iscove's Modified Dulbecco's medium
IMVS	Institute of Medical and Veterinary Science
LB	Luria Broth
LTR	long terminal repeat
mAb	monoclonal antibody
MAPK	Mitogen-activated protein kinase
MFI	Mean Fluorescence intensity
MML	Mo-MLV induced myeloid leukaemia
MQ water	Milli-Q purified water
mRNA	messenger RNA
MTHC	Myb transformed haemopoietic cell
mu	murine
N-terminus	amino-terminus
neo^R	neomycin resistance
NHS	normal human serum
NRS	normal rabbit serum
NSS	normal swine serum
OPC	Oligonucleotide purification column
PBA	PBS/BSA/Az
PBS	phosphate buffered saline

PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PI 3'-K	Phosphatidylinositol 3'-kinase
PKC	Protein kinase C
PLC-γ1	Phospholipase C-gamma 1
PNK	Polynucleotide kinase
RBC	red blood cells
RT	room temperature
RTK	receptor tyrosine kinase
RTPCR	Reverse transcribed PCR
SCF	Stem Cell Factor
SDS	Sodium dodecyl sulphate
SH	Src homolgy
Sl	*Steel*
SSC	Sodium Chloride / Sodium Citrate
SV40	Simian Virus 40
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TBS	Tris buffered saline
TC	tissue culture
TE	Tris-EDTA
TGF-β	Transforming growth factor- β
Tpo	Thrombopoietin
Tris	Tris(hyroxymethyl) amino-methane
W	*white spotting*
WBC	white blood cell

Chapter 1 : INTRODUCTION

1.1 ONCOGENES AND CANCER

The search for an understanding of the biochemical basis of cancer has focussed on the structure of oncogenes, regulation of their expression and the function of the products they encode. Oncogenes that cause cancer are altered versions of genes present in normal cells, and are now known to have originated from these cellular genes called "proto-oncogenes" (Bishop 1982).

1.1.1 Proto-oncogenes

The functions of the identified proto-oncogenes involve regulation of cell growth, division, proliferation, differentiation, maturation and death. Hence, the known proto-oncogenes are classified according to the physio-chemical role of their encoded products (reviewed in Varmus 1989 and references therein) into the following groups:

1. Kinases: enzymes involved in the phosphorylation of tyrosine, serine or threonine residues in proteins, as part of signal transduction within cells.
2. Guanyl-nucleotide binding proteins: proteins that are involved in the transmission of signals via GTPases (e.g. *ras*), and those that regulate the activity of GTP binding proteins.
3. Growth factors: extracellular factors that can bind to receptors on cells and initiate intracellular events.
4. Nuclear proteins: in particular those involved in regulating transcription of cellular genes.
5. Growth factor receptors: proteins that serve to bind extracellular factors and initiate intracellular signalling.
6. Anti-apoptotic factors: that immortalize cells by inhibiting cell death (e.g. *bcl-2*)

Research into oncogenes and their cellular progenitors has shed light on the basis of cancer, partly by increasing our understanding of the regulation of normal cellular activities. The proteins of oncogenes function abnormally, relative to that of their respective proto-oncogenes, and thereby induce "transformation" of a normal cell into a cancer cell.

1.1.2 The discovery of oncogenes

(a) Retroviruses

Oncogenes were first identified by the study of retroviruses. In 1911, Peyton Rous demonstrated that cell-free filtrates from chicken sarcomas could induce sarcomas in healthy chickens. The infectious agent was later identified as a virus designated Rous Sarcoma Virus (RSV) belonging to the class of RNA tumour viruses (now known as retroviruses). The critical factor, a gene called "*src*" which was carried by RSV was not identified until the early 1970's and it was in 1975 that a near exact copy of this gene was found in all chicken cells (reviewed in Hunter 1984). The study of retroviruses lead to the discovery of many known oncogenes and the identification of cellular proto-oncogenes some of which are shown in Table 1.1.

The mechanism of viral replication provides the basis for understanding the process of viral transduction of cellular genes. A schematic representation of RSV infection of a host cell and viral replication is shown in Figure 1.1 (Bishop 1982). Upon entering the host cell, the RNA genome of the retrovirus is transcribed into double stranded DNA by the viral enzyme reverse transcriptase. The DNA duplex or provirus may randomly integrate into host chromosomes and viral genes are expressed by host cell machinery. Two strands of genomic RNA, containing the gag, pol, and env genes are assembled together with retroviral enzymes in a virally encoded protein capsid. This complex forms a mature virion as it buds from the host cell using the cell

Table 1.1: Some of the proto-oncogenes transduced by retroviruses.

oncogene	retrovirus	species origin
Protein kinases		
src	RSV	chicken
yes	Y73-ASV	chicken
fgr	Gardner-Rasheed FeSV	cat
abl	HZ2-FeSV	mouse
	FuSV, PRCH-ASV,	chicken
fps/fes	Synder-Theilen FeSV	cat
mos	MSV	mouse
raf/mil	3611-MSV	mouse
ros	UR11-ASV	chicken
erb B	AEV	chicken
fms	SM-FeSV,HZ5-FeSV	cat
kit	HZ4-FeSV	cat
GTP binding proteins		
H-ras	Harvey-MSV	rat
K-ras	Kirsten-MSV	rat
Growth Factors		
sis	SSV	monkey
	Parodi-Irgens FeSV	cat
Nuclear proteins		
myc	Avian MC29 myelocytoma virus	mouse
myb	AMV, AMV-E26	chicken
fos	FBJ-MSV	chicken
ski	Avian SKV770 virus	mouse
rel	REV	turkey
ets-1	AMV-E26	chicken
Hormone Receptors		
erb A	AEV	chicken
unclassified		
cbl	murine Cas NS-1 leukaemia virus	mouse

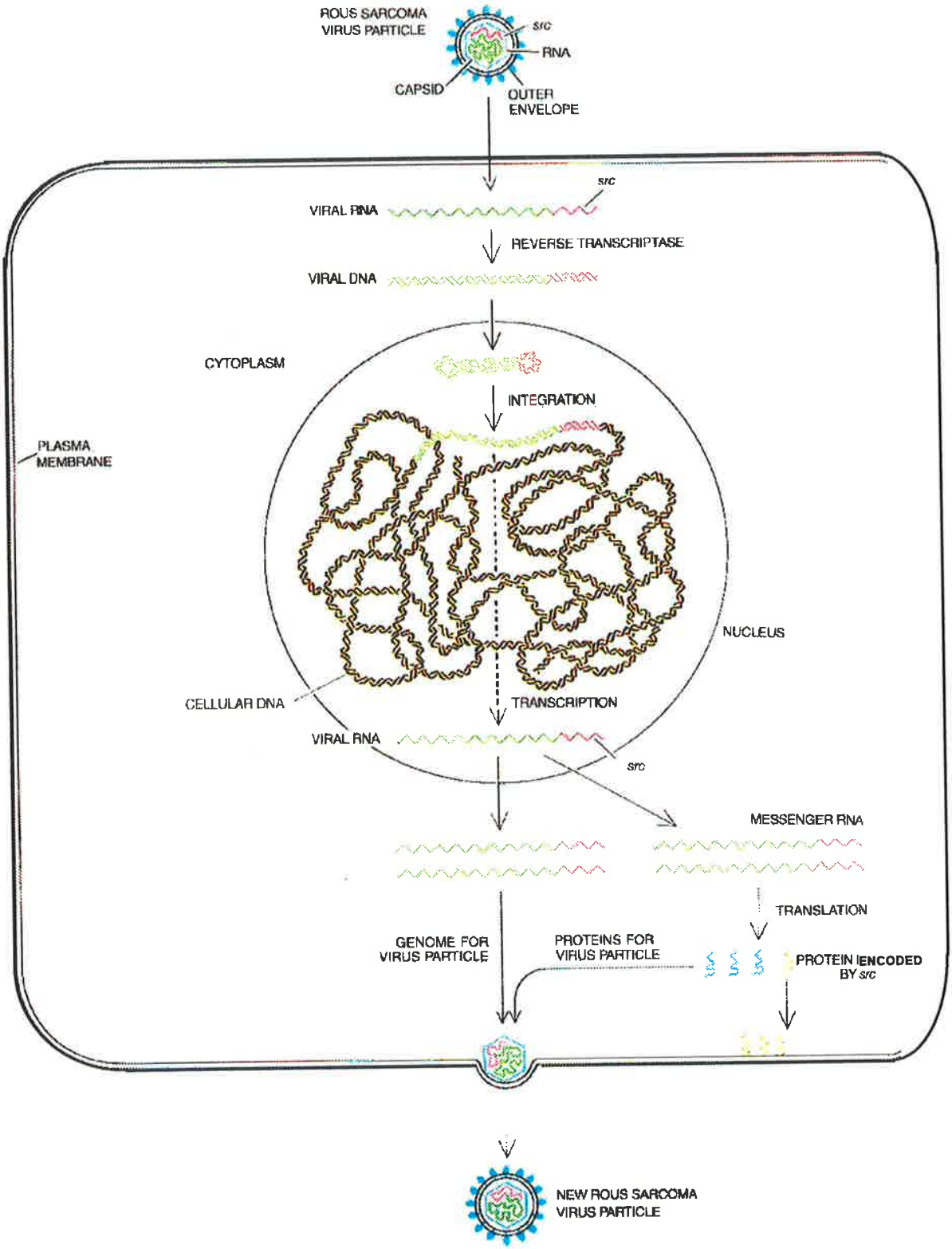
Abbreviations : (RSV) Rous Sarcoma Virus; (ASV) Avian Sarcoma Virus; (FeSV) Feline Sarcoma Virus; (FuSV) Fujinami Sarcoma Virus; (MSV) Moloney murine sarcoma virus; (AEV) Avian Erythroblastosis Virus; (SSV) Simian Sarcoma Virus; (AMV) Avian Myeloblastosis Virus; (REV) Reticuloendotheliosis Virus.

N.B. The information in this table was obtained from Bishop 1982; Bishop 1983; Bishop 1991; Hunter 1984; G. Caruana Ph.D. thesis - University of Adelaide.

Figure 1.1: Infection of a target cell by RSV.

The steps following infection include reverse transcription and integration. This leads to the expression of the virally encoded oncogene *src* in the target cell, as well as the production of progeny virus.

This figure is taken from Bishop, 1982.


ROUS SARCOMA VIRUS PARTICLE
src
RNA
CAPSID
OUTER ENVELOPE
VIRAL RNA
src
REVERSE TRANSCRIPTASE
VIRAL DNA
CYTOPLASM
INTEGRATION
PLASMA MEMBRANE
NUCLEUS
CELLULAR DNA
TRANSCRIPTION
VIRAL RNA
src
MESSENGER RNA
TRANSLATION
GENOME FOR VIRUS PARTICLE
PROTEINS FOR VIRUS PARTICLE
PROTEIN ENCODED BY src
NEW ROUS SARCOMA VIRUS PARTICLE

membrane as an outer coat (reviewed in Varmus 1989). Oncogenic retroviruses like RSV also contain genes that were acquired by viral transduction, a process in which a gene of host cell origin is incorporated into the viral genome. The acquired gene sequence becomes part of the viral RNA is therefore expressed in all subsequently infected cells. Usually, the cellular genes transduced are structurally altered during the integration event or contain mutations created by error-prone reverse transcription during subsequent infections. If these alterations in the cellular genes cause activation of their encoded proteins, the cells infected with retroviruses (carrying theses "oncogenes") become transformed resulting in tumours. Additionally, viral oncogenes also differ from their respective proto-oncogene by the regulation of their expression. They are expressed under the influence of strong, very efficient transcriptional signals provided by retroviral long terminal repeats (LTRs). For example, in the case of RSV (Figure 1.1), the protein encoded by v-*src,* although derived from c-*src*, is continuously expressed by strong viral promoters and hence is at a higher concentration in the cell. In addition, v-*src* also contains mutations that cause higher levels of protein phosphorylation and activity. These two differences of increased levels and stronger activity enable v-*src* to induce transformation of cells.

Retroviruses are not the only known tumour inducing viruses. DNA tumour viruses, especially the adenoviruses and papillomaviruses have also been the source of viral oncogenes (Varmus 1989). The simian virus 40 (SV40), for example, contains the genes encoding the large T antigen - a known transforming factor (Butel *et al.,* 1986). However, these genes are not transduced from host DNA, but rather are viral genes that perform mutiple functions, including the activation of viral and cellular promoters and binding to cellular proteins (e.g. SV40 large T complexes with cellular

p53) thereby causing disruption of the normal regulation of expression of cellular genes as well as altering the activity of cellular proteins.

Therefore, there are two underlying mechanisms that lead to oncogenesis : altered expression and / or activating mutations of cellular genes involved in regulating normal cellular activities. This suggests that virally encoded oncogenes are not essential for transformation and it has indeed been shown (as will be discussed below) that insertional mutagenesis by retroviruses resulting in cellular oncogenes can also lead to cancer.

(B) Cellular oncogenes (Proto-oncogenes)

The discovery of cellular oncogenes originated from an attempt to understand how viruses that lacked any oncogenes were able to cause tumours in animals. The Avian Leukosis virus (ALV), was able to cause a variety of diseases including lymphomas, but did not carry any oncogenes (reviewed in Varmus 1989). Examination of the ALV proviral DNA in various tumours showed integration at different positions within a single cellular locus (Neel *et al.,* 1981). Some tumours contained novel RNA in which viral regulatory sequences were joined to cellular sequences (Payne *et al.,* 1981). Molecular probes specific for retroviral oncogenes showed that the majority of lymphomas contained ALV proviral DNA in the c-*myc* locus (Hayward *et al.,* 1981; Payne *et al.,* 1982). This gene had already been identified as the cellular homologue of v-*myc*, an oncogene carried by several avian retroviruses able to cause acute myeloid leukaemias (AML)(Roussel *et al.,* 1979; Sheiness and Bishop, 1979). It is now known that altered expression of a proto-oncogene occurs in repetitive patterns in many cell lineages following proviral insertion due to infection by retroviruses, some of which are shown in Table 1.2. The mechanism of cellular transformation is very similar to that caused by retroviral

Table 1.2: Some of the proto-oncogenes activated by retroviral insertion.

gene	Insertion mutagen	Tumour type	species
c-myc	ALV,CSV,RPV	B-cell lymphoma	chicken
	Mo-MLV, MCF-MLV	T-cell lymphoma	rat, mouse
	FeLV	T-cell lymphoma	cat
c-erb B	ALV	erythroblastosis	chicken
c-fms	MLV	T-cell lymphoma	mouse
c-Ha-ras	MAV	nephroblastoma	chicken
	MLV	T-cell lymphoma	mouse
c-mos	IAP	plasmacytoma	mouse
c-myb	Mo-MLV	B-cell tumours	mouse
	ALV	B-cell lymphoma	chicken
int-1	MMTV	mammary carcinoma	mouse
int-2	MMTV	mammary carcinoma	mouse
int-3	MMTV	mammary carcinoma	mouse
int-4	MMTV	mammary carcinoma	mouse
pim-1	MCF-MLV, Mo-MLV	T-cell lymphoma	mouse
pvt/mis	Mo-MLV	T-cell lymphoma	mouse
lck	Mo-MLV	T-cell lymphoma	mouse
p53	Mo-MLV	erythroleukaemia	mouse
IL-2	GaLV	T-cell lymphoma	gibbon
IL-3	IAP	T-cell lymphoma	chicken

Abbreviations: (ALV) avian leukosis virus; (CLV) chicken syncytial virus; (RPV) ring-necked pheasent virus; (MAV) myeloblastosis-associated virus; (Mo-MLV) Moloney murine leukaemia virus; (FeLV) feline leukaemia virus; (IAP) intracisternal A particle; (MMTV) mouse mammary tumour virus; (MCF-MLV) mink cell focus-forming murine leukaemia virus.

N.B. This table is an updated version of one taken from Varmus 1989

oncogenes: the deregulation of expression of a particular cellular gene due to the infleuence of viral regulatory elements and or mutations in the coding sequence of the gene resulting in activation, both caused by proviral integration within the gene locus. One such oncogene is *myb* - it is carried as a viral oncogene by AMV (see Table 1.1) and has also been identified as a cellular oncogene activated by insertional mutagenesis following infection with ALV and MLV (see Table 1.2).

1.2 THE C-MYB PROTO-ONCOGENE AND IT'S PRODUCT

1.2.1 The discovery of c-myb - a historical perspective

Interest in the *myb* gene began with the identification of chicken leukaemias caused by two acute leukaemia viruses, AMV and the E26 virus (see Table 1.1, Moscovici and Gazzolo, 1982). Molecular cloning and nucleotide sequencing were used to determine the precise structures of the v-*myb* oncogenes of AMV and E26 (Roussel *et al.,* 1979; LePrince *et al.,* 1983; Rushlow *et al.,* 1982). This subsequently led to the identification of the homologous cellular proto-oncogene c-*myb*, as well as c-*myb* genes of other vertebrate species (Klempnauer *et al.,* 1982; Gonda and Bishop, 1983; Nunn *et al.,* 1984; Gerondakis and Bishop, 1986; Gonda *et al.,* 1985; Majello *et al.,* 1986). Sequence comparison of the viral and cellular genes established that the viral proteins (v-Myb) were truncated at their amino and carboxy termini with respect to cellular c-*myb* (Klempnauer *et al.,* 1982; Nunn *et al.,* 1984; Gerondakis and Bishop, 1986; see also Figure 1.2).

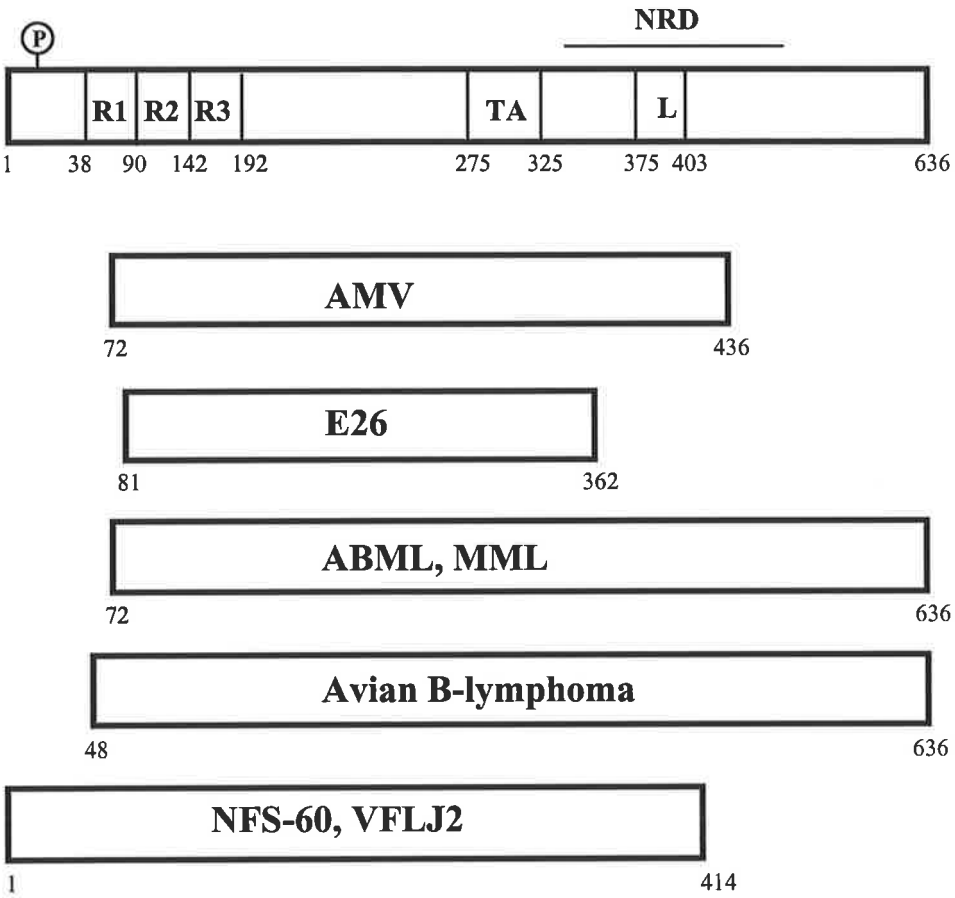
These v-Myb products exist as viral fusion proteins, namely the 48 kDa $p48^{v\text{-}myb}$ of AMV and the 135 kDa $p135^{gag\text{-}myb\text{-}ets}$ of E26 (Klempnauer *et al.,* 1982 and 1983) and their expression results in distinctive leukaemic phenotypes. The $p48^{v\text{-}myb}$ protein of AMV virus is responsible for monocytic leukaemias in chickens

Figure 1.2: Structural and functional domains of c-Myb and truncated forms of Myb.

The top bar shows the structural and functional motifs of full length c-Myb. 'P' represents the Casein kinase II phosphorylation site, 'R1-R3' represent the three tandem HTH motifs of the DNA-binding domain, 'TA' represents the domain required for the transactivation activity and 'NRD' represents the negative regulatory domain containing a leucine zipper-like motif labelled 'L'.

The other bars represent the truncated Myb proteins: encoded by retroviruses AMV and E26, expressed in virally induced leukaemias ABML and MML, and expressed in cell lines NFS-60 and VFLJ2.

This figure is taken from Gonda, 1991.


NRD
P
R1
R2
R3
TA
L
1
38
90
142
192
275
325
375
403
636
AMV
72
436
E26
81
362
ABML, MML
72
636
Avian B-lymphoma
48
636
NFS-60, VFLJ2
1
414

and for transformation of myeloid cells *in vitro* (Moscovici and Gazzolo 1982, Engelke and Lipsick 1994). On the other hand, the chimeric *gag-myb-ets* protein of E26 has been shown to be the causative agent of erythroleukaemias in chickens infected with this virus (Radke, 1982). This protein is responsible for the transformation of erythroid precursors *in vitro* (Nunn and Hunter, 1989; Metz and Graf, 1991a) although E26, like AMV, can also transform myeloid cells .

In addition to terminal truncations, the AMV v-*myb* gene contains 11 point mutations, with respect to the corresponding c-*myb* sequence. (Klempnauer *et al.*, 1983). However, these mutations and the viral sequences fused to the v-*myb* gene at either terminus are not required for transformation *in vitro* (Lipsick and Ibanez, 1987; Grasser, 1991). Similarly, the single point mutation in E26 v-Myb is not important for Myb-induced transformation (Nunn, 1983; Grasser, 1991). This suggests that truncation of the *myb* gene at either or both termini is the major determinant of oncogenic activation (reviewed in Gonda, 1991; Shen-Ong, 1990; Luscher and Eisenmann, 1990; see also the discussion below).

1.2.2 The c-*myb* gene

The cellular homologue of the v-*myb* genes is the c-*myb* proto-oncogene (see fig.1; Klempnauer and Bishop, 1983) . A single copy of the c-*myb* gene is present in the genome of widely divergent vertebrate species and sequences encoding the functional domains (see figure 1.2) are highly conserved (summarized in Shen-Ong 1990; Lipsick 1996). Two other vertebrate genes A-*myb* and B-*myb* share extensive homology with c-*myb* and are thought to have arisen from a common ancestor (Lipsick 1996). A-Myb functions as a transcriptional activator in male germ cells, a subset of B cells and in neuronal precursors. In contrast, B-Myb is more widely expressed there is controversy about whether it is a transcriptional activator or

repressor (Lipsick 1996). Both v-Myb and c-Myb proteins are nuclear localised (Klempnauer *et al.,* 1984) and have short half lives (Boyle *et al.,* 1984). Myb proteins bind DNA (Moelling *et al.,* 1985) and exhibit transactivation potential (Nishina *et al.,* 1989). Murine c-Myb is comprised of 636 amino acids (Gonda *et al.,* 1985) and can be divided into three functional domains responsible for DNA binding, transactivation and negative regulation as shown in Figure 1.2 (Sakura *et al.,* 1989). The DNA binding domain and the transactivation domain are essential for Myb activity.

(A) Myb binds DNA

Sequence comparison of Myb and the family of Myb-related proteins, reveals three tandem repeats R1-R3 (see Figure 1.2), each of 51 or 52 amino acids at the amino terminus (Gonda *et al.,* 1985; Klempnauer and Sippel, 1987). These repeats are highly conserved across many vertebrate species including Drosophila (Gerondakis and Bishop 1986, Gonda *et al.,* 1985, Frampton *et al.,* 1989, Majello *et al.,* 1986, Peters *et al.,* 1987). Each repeat folds into a helix-turn-helix (HTH)-like motif (Ogata *et al.,* 1993), a structure that is commonly found in eukaryotic transcription factors. A double HTH structure (R2 and R3) is necessary for DNA dinding by Myb (Gabrielson *et al.,* 1991, Klempnauer and Sippel 1986, Howe *et al.,* 1990, Oehler *et al.,* 1990). Mobility shift assays were used to show that Myb binds as a monomeric protein (Howe *et al.,* 1990) to the consensus sequence PyAACG/TG (where Py represents any pyrimidine) (Biedenkapp *et al.,* 1988; Nakagoshi *et al.,* 1990; Garcia *et al.,* 1991). Each HTH repeat contains three tryptophan residues spaced 18-19 amino acids apart , that are essential for the DNA binding activity of Myb proteins (Saikumar *et al.,* 1990; Kaneii-Ishii *et al.,* 1990). It has now been shown that these tryptophan residues as well as two histidine residues form a hydrophobic core which stabilizes the α helices of the HTH conformation, as was previously proposed (Frampton *et al.,* 1991;

Ishii *et al.,* 1992; Ogata *et al.,* 1992). It was found that R3, containing an intact HTH related structure, was important in recognising the central AAC sequence and R2 that supported this binding by interacting with phosphate groups of the DNA (Tanikawa *et al.,* 1992). Further reports on the NMR solution structures of the Myb/DNA complex, as well as the recognition helices involved in the interaction have provided more insight into the specificities of DNA binding (Ogata *et al.,* 1993; Ogata *et al.,* 1994; Morikawa *et al.,* 1995). A similar study has also shown that the hydrophobic core of the Myb DNA-binding domain is reserved for DNA recognition and trans-activation by Myb (Ogata *et al.,* 1996). Evidence indicates that Myb binds with varying affinity to different recognition sites and that binding is dependent on the sequence flanking the recognition site (Ramsay *et al.,* 1992).

Mutational analysis has also indicated that certain amino acids are involved in more specific protein-DNA interactions. Amino acid sequence alignment of the DNA binding motifs (called the basic region) of helix-loop-helix and leucine zipper proteins has indicated that Myb also has a "basic region" (Carr and Mott, 1991). It was predicted that a conserved cysteine residue within this region might be important for specific DNA binding (Carr and Mott, 1991), a prediction which has since been confirmed. This particular cysteine residue was shown to be essential for Myb DNA binding (Guehmann *et al.,* 1992), for transcriptional transactivation and transformation (Grasser *et al.,* 1992).

(B) Myb acts as a transcriptional activator

In experiments designed to further define the function of Myb, multiple copies of Myb binding sites (myb responsive elements or MREs) were placed upstream of a CAT reporter gene. Cotransfection of this CAT reporter plasmid, along with a *myb* expression plasmid, into cells resulted in activation of CAT expression (Sakura *et al.,*

1989). Additionally, deletion analysis was used to map the region responsible for transactivation. The transactivation domain , which is distinct from the DNA binding domain (Sakura *et al.,* 1989; Weston and Bishop, 1989; Lane *et al.,* 1990; see figure 1.2A), is hydrophilic and slightly acidic, a characteristic of many transcription factors (reviewed in Ptashne, 1988; Mitchell and Tijan, 1989). This, and other evidence, indicates that v-Myb and c-Myb are capable of functioning as transcription factors (Nishina, 1989; Sakura 1989; Klempnauer, 1989; Weston and Bishop,1989), which strongly suggests that, *in vivo,* Myb is involved in the regulation of expression of cellular genes (reviewed in Luscher and Eisenman 1990; Gonda 1991; Thompson and Ramsay 1995).

The most crucial, and to this point, most difficult, aspect of any investigation into the function of c-Myb, and the pathway to transformation by v-Myb, is that of determining the identity of the genes that are transactivated by Myb *in vivo*. The *mim-1* gene has been shown to be directly transactivated by Myb (Ness *et al.,* 1989), but its function is unknown. Two genes whose products are associated with DNA replication have been reported to be regulated by c-Myb. These are DNA polymerase α and proliferating cell nuclear antigen (PCNA), (Venturelli *et al.,* 1990). The expression of several other genes, including the c-*myc* proto-oncogene (Evans *et al.,* 1990; Zobel *et al.,* 1991, Cogswell *et al.,* 1993), the c-*kit* oncogene (Ratajczak *et al.,* 1992), the CD4 gene (Siu *et al.,* 1992), the IL-2 gene (Rose and Reddy, 1992) and the cdc2 gene (Ku *et al.,* 1993) have also been linked to Myb activity.

There is now increasing evidence that Myb combines with other transcription factors to activate specific genes. v-Myb has been shown to act synergistically with different members of the C/EBP family of transcription factors in the activation of the mim-1 gene in mammalian myelomonocytic cells (Burk *et al.,* 1993). In chicken

myeloid cells, the NF-M factor, thought to be the avian homologue of mammalian C/EBPβ, was shown to be required together with Myb for the full activity of the mim-1 gene (Ness *et al.,* 1993). NF-M is normally expressed in myeloid cells, but ectopic expression of MYB and NF-M in erythroid cells or fibroblasts, induced expression of endogenous mim-1 and lysozyme genes, markers of myeloid differentiation (Ness *et al.,* 1993). More recently, CBP (CREB binding protein) has been implicated as a co-activator of Myb (Dai *et al.,* 1996), possibly by providing a bridge between Myb and other factors like NF-M (Oelgeschlager *et al.,* 1996). Therefore, tissue-specific factors and other co-regulators that bind to Myb can have a strong influence on Myb activity by co-operatively regulating the expression of specific target genes. However, another study showed that the regulation of c-*myc* expression by Myb did not require the *de novo* synthesis of additional protein factors or the presence of other labile proteins, but still varied between different cell lineages (Cogswell *et al.,* 1993). Besides the above evidence which suggests that co-activators can influence Myb function, other mechanisms have been shown to regulate Myb activity.

(C) The leucine zipper motif and other regulatory sites

The most striking feature of the negative regulatory domain (Lane *et al.,* 1990, Sakura *et al.,* 1989), is the leucine zipper motif (see Figure 1.2A). This domain is deleted in v-*myb* of E26 (see Figure 1.2B), and mutant c-Myb proteins that contain C-terminal truncations or deletions of the leucine zipper motif exhibit transforming capacity (Gonda *et al.,* 1989b, Hu *et al.,* 1991). These mutant forms of Myb also show increased DNA binding (Ramsay *et al.,* 1991) and increased transcriptional activity (Sakura *et al.,* 1989, Kaneii-Ishii *et al.,* 1992). Furthermore, specific mutations in the leucine residues that are believed to be necessary for maintaining the structure of this motif cause an increase in transactivation and transformation capacity of c-Myb

(Kaneii-Ishii *et al.,* 1992). This indicates that the leucine zipper structure plays an important role in the regulation of c-Myb activity and disruption of this motif enhances the transforming capacity of Myb. This motif has been shown to mediate the binding of other cellular proteins (Favier and Gonda, 1994) and is believed to negatively regulate Myb by allowing associations with other factors that in turn alter or inhibit its transactivation capacity (Ramsay *et al.,* 1995). Another motif in the NRD of Myb that has been postulated to be important in Myb activity is the EVES motif which is also highly conserved between species. The EVES motif has been shown to be a substrate for phosphorylation, and mutations within this sequence result in an increase in the trans-activation capacity of Myb (Aziz *et al.,* 1993; Aziz *et al.,* 1995). A ubiquitously expressed transcriptional co-activator p100 also contains an EVES motif and both the EVES motifs from Myb itself and p100 have been shown to be able to bind to the DNA-binding domain of Myb (Dash *et al.,* 1996). It has been proposed that binding of p100, via its EVES motif, to the DNA-binding domain of Myb mediates interactions between Myb and other cellular co-factors, thereby maintaining Myb in an active state. Conversely, the binding of the EVES motif within Myb to its own DNA-binding domain, prevents interactions between Myb and other cellular co-factors, maintaining Myb in an inactive state (Dash *et al.,* 1996). This suggests that specific intramolecular interactions and conformational changes could control the interaction between Myb and the transcriptional apparatus or co-factors, and hence regulate the activity of Myb.

Other regions within the Myb protein have also been shown to play a role in regulation by modulating the DNA binding of Myb and in turn its activity. In the amino terminal region, phosphorylation of the Casein Kinase II site (see Figure 1.2A), has been shown to positively modulate DNA binding as well as transactivation

(Oelgeschlager *et al.,* 1995, Ramsay *et al.,* 1995). Although the first repeat in the DNA binding domain is not essential for DNA binding, truncation of this repeat has been shown to decrease DNA binding by Myb (Dini and Lipsick 1993). The linker regions between the HTH repeats, that are highly conserved in vertebrate species, have also been shown to play a role in stabilizing DNA binding (Hegvold *et al.,* 1996).

Due to the role of c-Myb as a transcriptional activator, any structural alterations in the protein that cause variation in the activity of Myb, also lead to changes in its biological function.

1.3 C-MYB FUNCTION AND ACTIVATION

1.3.1 The biological role of c-Myb

To date, there is a large body of evidence linking c-Myb to the proliferation of early haemopoietic cells. For example, high levels of c-*myb* transcripts and proteins have been observed in proliferating, immature haemopoietic cells and these levels fall markedly as the cells differentiate (Gonda *et al.,* 1982; Westin *et al.,* 1982; Gonda and Metcalf, 1984). This pattern is detected in myelomonocytic, erythroid and B and T lymphoid cells analysed in humans, chickens and mice (Westin *et al.,* 1982; Gonda *et al.,* 1982; Duprey and Boettinger 1985). Correspondingly, tissues active in haemopoiesis, such as the bone marrow and foetal liver in mice and the yolk sac, thymus and bursa in chickens, exhibit the highest levels of expression of c-*myb*, (Gonda *et al.,* 1982). Additionally, induction of T-lymphocyte proliferation by IL-2 is accompanied by a transient increase in the levels of c-*myb* expression (Stern and Smith, 1986; Lipsick and Boyle, 1987).

Proliferation of normal haemopoietic cells, HL-60 leukaemic cells and CML blast cells, *in vitro*, can be inhibited by using c-*myb* antisense oligonucleotides to reduce levels of c-*myb* expression (Gerwitz and Calerbretta, 1988; Anfossi *et al.*, 1989; Venturelli *et al.*, 1990; Citro *et al.*, 1994; Gerwitz 1993a, 1993b). *In vivo* studies showed that c-myb antisense oligonucleotides decreased the long term growth of human melanomas, and human leukaemias in SCID mouse models (Ratajczak *et al.*, 1992; Gewirtz, 1993a, 1993b; Hijiya *et al.*, 1994). Furthermore, inactivation of c-*myb* by homologous recombination results in a large reduction of foetal liver haemopoiesis in transgenic mice and subsequent death of the foetus early in its development (Mucenski *et al.*, 1991). Additionally, differentiation in various leukaemic cell lines can be blocked by enforcing over-expression of c-*myb* (Clarke *et al.*, 1988; M^{c}Clinton *et al.*, 1990; Yanagisawa *et al.*, 1991; Selvakumaran *et al.*, 1992; Cuddihy *et al.*, 1993; Bies *et al.*, 1995). Overall, these observations indicate that c-Myb is a key regulator of haemopoietic cell proliferation and differentiation, and the evidence suggests that c-Myb is essential for maintaining the proliferative state of certain haemopoietic progenitor cells (reviewed in Gonda, 1991; Shen-Ong, 1990; Luscher and Eisenman, 1990). The function of Myb as a regulator is altered by activation through over-expression and structural alteration.

When activated, Myb is able to cause transformation of cells into a state of continuous proliferation and to enforce a block in their differentiation (reviewed in Gonda, 1991; Thompson and Ramsay, 1995). The truncated v-Myb proteins of AMV and E26 transform avian myeloid cells into an immature continuously proliferating state and cause tumours *in vivo* (Gazallo *et al.*, 1979; Beug *et al.*, 1979; Durban and Boettiger *et al.*, 1981). Activation of c-*myb* by proviral integration is also known to

lead to tumours due to continuous proliferation and a block in differentiation as described below.

1.3.2 Activation of c-*myb* by proviral insertion

Amino and carboxy terminal truncations of the c-Myb protein, due to retroviral integration within the c-*myb* gene, have been reported in over thirty cell lines derived from tumors induced by retroviral infection (Shen-Ong *et al.,* 1984; Shen-Ong *et al.,* 1986; Shen-Ong and Wolff, 1987; Weinstein *et al.,* 1987; Gonda *et al.,* 1987; Kanter *et al.,* 1988; Mushinski *et al.*, 1983; Pizer and Humphries, 1989; reviewed in Wolff *et al.,* 1991; Wolff *et al.,* 1996; Wolff, 1997). Tumours induced by infection with Abelson murine leukaemia virus (Ab-MLV) possess high levels of aberrantly sized *myb* transcripts. (Mushinski *et al.,* 1983). These monocytic tumours, originally termed ABPLs (Ab-MLV induced plasmacytoid lymphosarcomas), expressed high levels of amino terminally truncated c-Myb (see Figure 1.2B), due to proviral insertion into the cellular c-*myb* gene (Lavu and Reddy, 1986) by the helper virus Moloney murine leukaemia virus (Mo-MLV) (Shen-Ong *et al.,* 1984; Gonda *et al.,* 1987; Shen-Ong and Wolff, 1987). Similar tumours can be induced by infection with Mo-MLV alone and are termed MMLs (Mo-MLV induced myeloid leukaemias), (Shen-Ong *et al.,* 1986). These tumours have arisen by insertion of retroviral DNA into the 5' region of the c-*myb* gene, resulting in high levels of transcription from the strong promoter in the viral long terminal repeat (LTR) . In these tumours, viral splicing signals generate a hybrid c-*myb* transcript encoding amino terminally truncated Myb proteins. (Shen-Ong and Wolff, 1987; Shen-Ong *et al.,* 1986 and Gonda *et al.,* 1987). Some B-cell tumours induced by infection of chick embryos with avian leukosis virus (ALV) (Pizer and Humphries, 1989; Kanter *et al.,* 1988), show similar sites of integration to those of ABML and MML and the Myb

proteins expressed in these cells are also truncated at their amino termini. (see Figure 1.2B; Moscovici and Gazzolo, 1982). Murine myeloid cell lines have also been isolated in which expression of c-Myb truncated at the carboxy (C)- terminal was detected (Shen-Ong *et al.,* 1986). This was caused by retroviral insertion downstream within the c-*myb* locus which resulted in early termination of transcription within the viral LTR. The C-terminally truncated forms of Myb expressed in two specific cell lines NFS-60 (Lavu and Reddy 1986) and VLF-J2 (Weinstein *et al.,* 1987) are shown in Figure 1.2. These data suggest that activation of Myb is associated with constitutive high level expression and/or terminal truncation (reviewed in Gonda, 1991 and Shen-Ong, 1990).

1.3.3 Oncogenic activation and transformation

As discussed previously, c-Myb is a known regulator of growth and differentiation of erythroid, lymphoid and myeloid cells (reviewed in Gonda 1991) as well as some non-haemopoietic cells (reviewed in Thompson and Ramsay, 1995). Correspondingly, infection by AMV can lead to the development of B cell lymphomas in chickens and activated forms of murine c-Myb result in factor-dependent transformation of murine myeloid cells (reviewed in Gonda 1991). These activated forms of Myb differ in expression levels and structure when compared to c-Myb. The oncogenic forms of Myb are continuously expressed from LTR promoters and are terminally truncated (see Figure 1.2). The v-Myb proteins encoded by AMV and E26 are truncated at both termini, with respect to the chicken c-Myb protein (Klempnauer *et al.,* 1982; Nunn *et al.,* 1984; Gerondakis and Bishop, 1986). Structural alteration of c-Myb, in the form of terminal truncation of the protein, and its over-expression, have also been detected in a number of cell lines that have arisen due to cellular transformation caused by retroviral integration, as discussed earlier. *In vitro*

studies with foetal liver cells (FLC) have shown that disruption of the negative regulatory domain (Sakura *et al.,* 1989) by C-terminal truncation or mutations in the leucine zipper structure (Kanei-Ishii *et al.,* 1992) has resulted in transformation of primary haemopoietic cells, as determined by clonogenic assays and the generation of long term cell lines (Gonda *et al.,* 1989b; Hu *et al.,* 1991). Together, these observations suggest that high levels of structurally altered activated forms of Myb can cause cellular transformation (reviewed in Shen-Ong 1990 and Gonda 1991), but how this occurs is yet to be understood.

It is known that both the DNA binding and the transactivation domains of Myb are required for the transformation of haemopietic cells (Moelling *et al.,* 1985; Lane *et al.,* 1990; Hu *et al.,* 1991) and to block the differentiation of leukaemic cells (Cuddihy *et al.,* 1993). Some mutant forms of v-Myb have been identified that are capable of transactivation, but as yet have not been shown to exhibit transforming potential (Chen and Lipsick 1993). Recently, it was shown that transcriptional activation required cooperation among multiple domains including a central, C-terminal and at least two N-terminal domains (Chen *et al.,* 1995). It was also shown that although various subsets of these domains were sufficient for transcriptional activation, all the domains were necessary for transformation of haemopoietic cells (Chen *et al.,* 1995). Therefore, it is now clear that transactivational capacity alone is not sufficient to result in transformation.

In a separate study, experiments indicated that although the transactivation domain of Myb was essential for transformation, substitution of the HSV-1 VP16 transactivation domain for that of Myb could restore transforming potential and there were no differences observed in the characteristics of the transformed cells (Frampton *et al.,* 1993). This showed that although the transactivation capacity of Myb was

required for function as a transforming factor, the specific transactivation domain of Myb did not seem to be important for Myb function. It is possible that the other domains mentioned above (Chen *et al.,* 1995) could be important in providing the specificity of trasactivation and hence transformation.

Carboxy terminal truncation of the c-Myb protein that results in an increase in the transactivation of reporter gene expression as mentioned previously (Sakura *et al.,* 1989) also results in a parallel increase in its capacity to transform haemopoietic cells (Gonda *et al.,* 1989a; Gonda *et al.,* 1989b; Hu *et al.,* 1991). Similarly, mutations in the leucine zipper region of c-Myb that cause an increase in transactivation of a reporter gene also result in a corresponding increase in transformation ability (Kanei-Ishii *et al.,* 1992). All these results indicate a definite correlation between the transactivation capacity and the transformation potential of structurally altered forms of murine Myb, as is the case with avian Myb (Lane *et al.,* 1990).

Taken together, all these results discussed above indicate that Myb causes transformation of cells by acting as a transcriptional activator, and that although transactivating capacity alone seems to be insufficient for transformation, an increase in the transactivating capacity of activated forms of Myb correlates with transforming capacity. Additionally, DNA binding activity, affinity or specificty (see section 1.2.2 (C)) of Myb are known to be important for function. It is possible that changes in any of these due to oncogenic mutation, could modify Myb activity, by altering the targets of transactivation, and hence lead to transformation. However, the exact mechanism by which oncogenic activation by structural alteration results in transformation is still unknown and some assumptions with regard to transformation by Myb that have yet to be experimentally addressed. One such assumption is that structural alteration of Myb is necessary for activation and the transforming potential of Myb. It was unclear

whether over-expression alone was sufficient to cause transformation. This was investigated and is described in Chapter 3.

Another assumption, is that activation of Myb alone is insufficient for the development of a malignancy *in vivo* and that other co-operative factors (activated oncogenes) may be also required. Murine haemopoietic cell lines obtained by Myb induced transformation are factor-dependent *in vitro* and become tumourigenic following the loss of factor-dependence (Gonda *et al.,* 1989a). Additionally, a number of *in vivo* murine models have shown that activated forms of Myb can be detected prior to the development of any haemopoietic malignancy suggesting that activation of Myb is an early step in leukaemia (Nason-Burchenal and Wolff, 1993; Belli *et al.,* 1995). In these studies on the murine system and others with the avian system (Press *et al.,* 1995), which will be discussed further in chapter 4, the most consistent observation is that expression of activated forms of Myb can lead to but does not guarantee the development of a malignant phenotype. This issue was investigated further and results from a murine *in vivo* investigation are discussed in chapter 4.

Therefore, the underlying question arises as to whether or not over-expression of an activated form of Myb in haemopoietic cells can give rise to any kind of haemopoietic malignancies in animals. If additional co-operative factors are necessary for transformation initiated by Myb, ideal candidates would seem to be continuously expressed growth factors or constitutively activated growth factor receptors. Another possible candidate, also a known proto-oncogene is the receptor tyrosine kinase, c-Kit.

1.4 THE C-KIT PROTO-ONCOGENE AND ITS PRODUCT

1.4.1 The discovery of c-Kit - a historical perspective

A feline leukaemia virus (FeLV) belonging to the Hardy Zucherman 4 strain

(HZ4-FeSV) present in filtrate obtained from a primary feline fibrosaroma was shown to transform feline embryonic fibroblasts and mink cells (Besmer *et al.,* 1986). Analysis of the proviral DNA of HZ4-FeSV which was not homologous to FeLV, enabled the isolation of a 1.1kb non-viral sequence designanted v-*kit*, the product of which was a gag fusion protein. Sequence homology was found in DNA from cat, human and mouse cells, and in all cases the transcript size ranged from 5-5.5kb (Yarden *et al.,* 1987; Qui *et al.,* 1988). The cellular proto-oncogene of v-*kit* was cloned from mouse brain and human placenta libraries. The v-*kit* gene product is truncated at both termini with respect to the c-*kit* gene, shown in Figure 1.3, and also contained differences in certain amino acids. Most variations were shown to be divergence between species except for two specific deletions at Tyr569 and Val570 located within a putative tyrosine substrate interaction site, that have been shown to contribute to oncogenic activation (Herbst *et al.,* 1995b). The v-Kit protein is attached to the membrane via a gag-linked myristoylation as it lacks the entire extracellular domain, the transmembrane domain and the first 17 amino acids as well as the last 49-50 amino acids of the intracellular domain. The functional domains of Kit, as well as activating mutations, will be discussed further in the following sections.

The human c-*kit* cDNA encodes a 976 amino acid polypeptide of 125 kD that is glycosylated giving rise to a 145 kD functional protein. c-Kit shares major homology with CSF-1 receptor also known as c-Fms, schematically represented in Figure 1.3A (reviewed in Yarden and Ullrich 1988) as well as the newly characterised flt3/flk2 receptor (Matthews *et al.,* 1991; Rosnet *et al.,* 1991). The major differences between the proteins lie in the amino acid sequences that give rise to the ligand binding domain and the kinase insert. It is these regions that provide the functional specificity of each receptor. By the structural homology between various receptors of

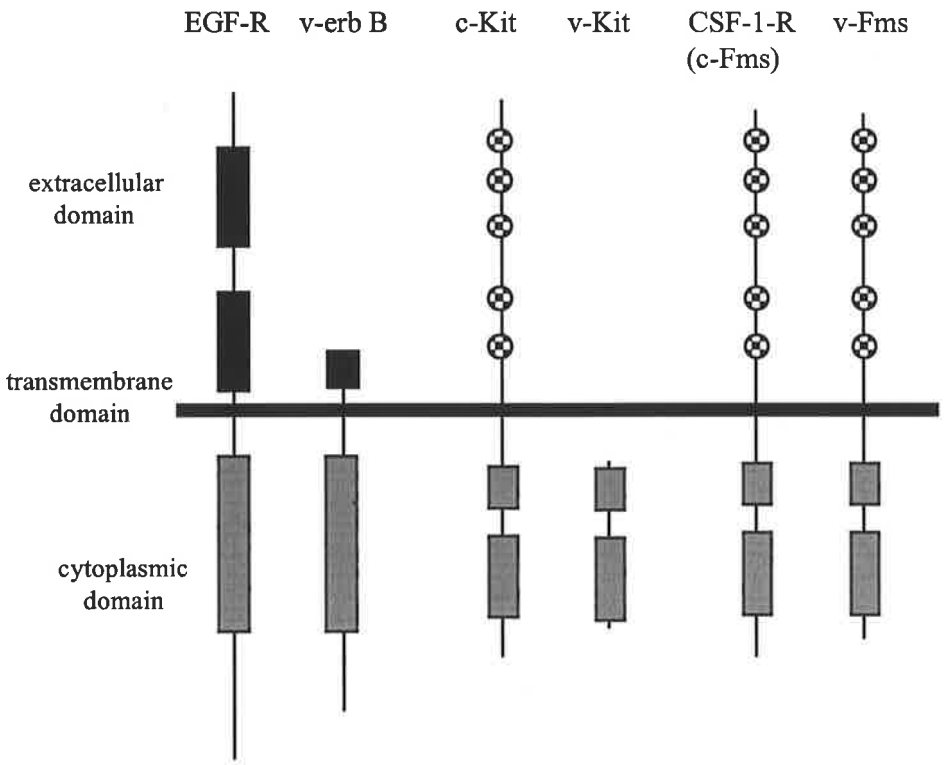
Figure 1.3: Structures of RTK proteins.

The dark boxes and the shaded circles in the extracellular region represent Cystein-rich repeats and Ig-like domains respectively. The light shaded boxes in the intracellular region represents tyrosine kinase domains.

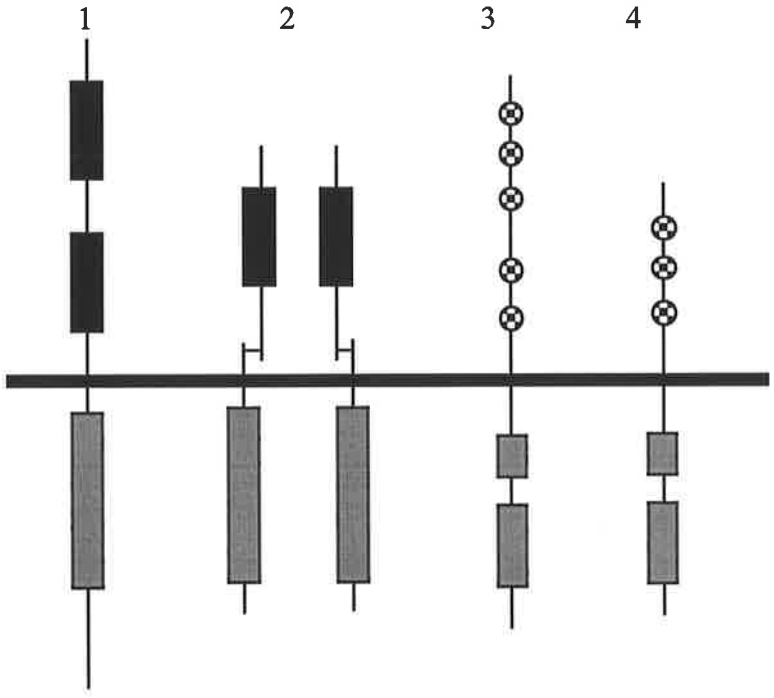
A: The proto-oncogenic and truncated oncogenic forms of three members of the RTK family. This figure is taken from Yarden and Ullrich, 1988.

B: The four RTK sub-classes and examples of members in each group. This figure is taken from Ullrich and Schlessinger, 1990.

A



B



1	2	3	4
EGF-R	Insulin-R	PDGF-R-A	FGF-R
HER2 / neu	IGF-1-R	PDGF-R-B	flg
HER3 / c-erbB-3	IRR	CSF-1-R	bek
Xmrk		c-Kit	
		flt-3	

the receptor tyrosine kinase (RTK) family, the proteins are grouped together in different sub-families shown in Figure 1.3B.

1.4.2 RTK : Structure and Function

All growth factor receptors belonging to the RTK family contain the following conserved features : a glycosylated extracellular ligand binding domain, a hydrophobic transmembrane domain and a cytoplasmic domain containing tyrosine kinase catalytic sites (reviewed in Ullrich and Schlessinger, 1990). New members of this family are continually being discovered (reviewed in Lemmon and Schlessinger, 1994).

Upon binding of its respective ligand, each RTK functions by catalysing the phosphorylation of the tyrosine residues within the receptor itself, followed by phosphorylation of exogenous substrates. The mechanism by which this occurs varies between the different subfamilies, but the sequence of events is the same in all cases. Ligand binding induces association between the receptor molecules that results in transphosphorylation and enables the phosphorylation of other cellular substrates by the activated receptor, initiating the signalling cascade (reviewed in Ullrich and Schlessinger, 1990).

The RTK 1 family (see Figure 1.3B) proteins possess two cysteine-rich repeats that are involved in binding ligand. Dimerization of receptors leads to phosphorylation and activation. RTK 2 proteins function as heterotetrameric structures involving two α and β subunits linked by disulphide bonds. The α subunits with cysteine-rich repeats are involved in ligand binding and the β subunits possess tyrosine kinase activity. RTK 3 proteins have five immuloglobulin (Ig) -like domains in the extracellular domain, which are involved in binding of a dimeric ligand, and a split intracellular tyrosine kinase domain. Ligand binding initiates activation of the

receptor via autophosphorylation of the Tyr residues in the tyrosine kinase domain. RTK 4 proteins are similar to RTK 3 proteins except that they contain fewer extracellular Ig-like domains.

Many substrates involved in the signalling cascade initiated by phosphorylation of the receptors are shared by members of the RTK family (reviewed in Schlessinger 1995; Ullrich and Schlessinger 1990; Cantley *et al.,* 1991). The majority of the subtrates bind to phosphorylated tyrosine residues on the activated receptors. Some known subtrates contain Src homology 2 (SH2) domains. The mechanism of the diverse functions of the different receptors in spite of the overlapping signal pathways is yet to be completely understood. It is possible that the level of receptor, the duration of the signal or even the relative concentations of substrates could provide specificity of signalling. Since the field of research into signalling by receptors is extensive as well as redendant in some cases, the focus of this discussion will now be on c-Kit activity.

1.4.3 Expression of the c-Kit gene

In murine and human genomes, c-*kit* spans over 80kb with the coding sequence encompassing 21 exons (Andre *et al.,* 1992; Giebel *et al.,* 1992;Gokkel *et al.,* 1992; Vandenbark *et al.,* 1992). By *in situ* hybridization, the human c-*kit* gene was mapped to the long arm of chromosome 4 between 4q11-q21 and murine c-*kit* to chromosome 5 (Yarden *et al.,* 1987), linking c-*kit* to the White-spotting (W) locus in mice. Mutant W mice provided an insight into the function of c-Kit and will be discussed further in section 1.5. There are two alternative splice variants of the c-kit mRNA due to the use of an alternative 5' splice donor site within exon 9 (that encodes the transmembrane domain). These two messages either contain (Kit+ / long form / GNNK+) or lack (Kit A+ / short form / GNNK-) 12bp encoding animo acids

Gly,Asn,Asn,Lys in the extracellular domain immediately prior to the transmembrane domain (Hayashi *et al.,* 1991, Reith *et al.,* 1991, Gokkel *et al.,* 1992, Vandenbark *et al.,* 1992). Both isoforms of Kit are expressed in placenta, foetal liver, lung, liver, ovary, testis, melanocytes, glioblastoma cells, normal bone marrow (BM) cells, leukaemic cell lines and Acute Myeloid Leukaemia (AML) cells (Reith *et al.,* 1991, Giebel *et al.,* 1992, Crosier *et al.,* 1993, Piao *et al.,* 1994, Zhu *et al.,* 1994). In humans, due to another alternative splice site, there are also two other naturally occuring human c-Kit isoforms, one that contains and one that lacks 3bp encoding a Ser residue at amino acid 715 in the inter-kinase domain (Crosier *et al.,* 1993). These two isoforms are expressed in normal BM cells, leukemic cell lines and AML cells. The normal roles of these different isoforms is still uncertain, however they have been shown to exhibit different transformation potentials when over-expressed in fibroblasts (Gina Caruana, 1996: PhD thesis, University of Adelaide). All forms of naturally occurring murine c-Kit do not contain this Ser residue (Qui *et al.,* 1988).

Kit expression varies during different stages of embryogenesis and in the adult has been detected in haemopoietic, melanoblastic and germ cell lineages. The pattern of expression in the embryo and the adult seems to reflect the function of c-Kit in the tissues of the various systems. Monoclonal antibodies (mAB) against human c-Kit, used to detect cell surface expression on haemopoietic cells, showed that 1-4% of normal BM mononuclear cells were positive, including early progenitor cells and lineage restricted progenitor cells, as well as mature mast cells (Mayrhofer *et al.,* 1987; Cambareri *et al.,* 1988; Ashman *et al.,* 1991; Buhring *et al.,* 1991; Papayannopoulou *et al.,* 1991; Simmons *et al.,* 1994). This expression pattern is similar to that of Kit in the murine haemopoietic system (Ogawa *et al.,* 1991).

1.4.4 The c-Kit product and activation by its ligand SCF

Research on W mice, which are now known to have inactivating mutations in the c-*kit* gene, and Sl mice ~~which are~~ now known to have inactivating mutations in the gene encoding the ligand for c-Kit, had provided a basis for understanding the function c-Kit even prior to its identification or that of its ligand.

Mutations in the W locus affect normal haemopoiesis resulting in macrocytic anaemia, mast cell deficiencies, defects in the proliferation and migration of primordal germ cells (thereby affecting fertility), and result in the loss of hair pigmentation ("white spotting") due to defects in melanoblasts (reviewed in Sarvella and Russell 1956; Russell 1979). Many independent W mutations differing in their severity and their effects on different cell lineages have been identified, with some mutations being lethal. DNA and protein analysis of W mast cells revealed structural alterations, in the form of deletions and point mutations, within the tyrosine kinase domain of c-Kit, some which caused impairment of the kinase activity (Giessler *et al.,* 1988; Nocka *et al.,* 1989, 1990b; Rieth *et al.,* 1990). With variations in intracellular signalling by the different W mutants, it was suggested that a diversity of normal Kit signalling pathways existed (Reith *et al.,* 1990).

Mutations in the Sl locus of mice give rise to a similar phenotype to the W mutations. The Sl mutant mice exhibit moderately severe macrocytic amaemia, lack of pigmentation, a mast cell deficiency and sterility (Silvers 1979). In a similar manner to the W mutations, Sl mutations also vary in their effects on different cell lineages and in their severity. The effect of W mutations on the haemopoietic system can be rectified by the transplantation of wild type BM stem cells from normal donors, but the effects of Sl mutations may be remedied only by transplantation of normal stroma.

The Kit ligand was isolated from the conditioned medium of Balb/c fibroblasts based on its ability to stimulate proliferation of BM mast cells from normal mice but not W mutant mice (Nocka *et al.,* 1990a). The gene encoding the ligand was mapped to the Steel (Sl) locus on chromosome 10 in mice (Copeland *et al.,* 1990; Huang *et al.,* 1990; Zsebo *et al.,* 1990a) and to chromosome 12 in humans (Anderson *et al.,* 1991). The ligand was isolated from several different sources and consequently given various names including, Kit ligand (KL)(Nocka *et al.,* 1990a), mast cell growth factor (MGF)(Anderson *et al.,* 1990; Boswell *et al.,* 1990; Williams *et al.,* 1990), Steel factor (SLF)(Williams *et al.,* 1992) and Stem Cell Factor (SCF)(Zsebo *et al.,* 1990b). The ligand will be refered to as SCF throughout this thesis. SCF cDNA encodes a polypeptide containing a secretion signal peptide, an extracellular domain, a hydrophobic transmembrane domain and an intracellular domain (Martin *et al.,* 1990; Huang *et al.,* 1990; Anderson *et al.,* 1990). In the murine and human system, alternative splicing results in the expression of either a 248 or 220 amino acid protein. (Martin *et al.,* 1990; Lu *et al.,* 1991; Pandiella *et al.,* 1992). As the smaller form lacks the recognition site for a serine protease, cleavage of human SCF occurs only with the larger isoform, resulting in the production of a 164-165 amino acid soluble SCF protein. Proteolytic cleavage to produce soluble SCF occurs at a different site in the murine system. Both isoforms of murine SCF are cleaved at the same site, but each isoform is cleaved by a different protease (Huang *et al.,* 1992). Activation of, and signalling by, the c-Kit receptor are triggered by the binding of SCF, in the membrane bound or soluble form. It has been suggested that membrane bound and soluble SCF may have different roles in normal haemopoiesis, however the exact nature of any differences are as yet unknown.

(A) SCF binds to c-Kit

In the RTK family, the conserved motifs in the extracellular domain are involved in ligand binding (reviewed in Lemmon and Schlessinger 1994). Experiments using chimeric proteins of the murine and human c-Kit domains were used to identify the ligand binding site since, murine SCF is able to bind to both murine and human c-Kit receptors, but human SCF does not show high affinity binding to murine c-Kit (Martin *et al.,* 1990; Lev *et al.,* 1993). Additionally, the epitopes recognised by mAbs able to block SCF binding to c-Kit have been used to identify specific regions in the receptor that are important in associating with SCF (Lev *et al.,* 1993). Similarly, various soluble ectodomains used in binding experiments demonstrated the specific affinities of the receptor domains to associate with ligand (Blechman *et al.,* 1993a,b). Together the results showed that the second Ig domain of c-Kit was directly involved in binding SCF, Ig domain one contained sites for high affinity binding of SCF and Ig domain three was important in the formation of the ligand binding cleft. This suggested that domain two was critical for SCF binding while domains one and three were important in stabilising the association necessary for high affinity binding (Blechman *et al.,* 1993a,b; Lev *et al.,* 1993).

It was initially demonstrated that SCF could bind to Kit (in a monovalent or bivalent manner) inducing Kit receptor dimerization and activation (Blume-Jensen *et al.,* 1991; Lev *et al.,* 1992b). It has now been confirmed that soluble SCF can exist as a non-covalently associated dimer and is able to form a complex with two molecules of the Kit receptor (Philo *et al.,* 1996). c-Kit can be activated by both membrane bound and soluble SCF, however, membrane bound SCF is able to induce a more persistent activation of the receptor kinase and a longer lifespan of the Kit protein on the surface (Miyazawa *et al.,* 1995). This is thought to be due to prevention of

internalisation of the Kit/SCF complex, a process known to result in down-regulation of surface receptor Kit, which is induced following activation by soluble SCF (see section 1.4.4(C)).

Ligand binding induces homo-dimerization of the receptor, which in turn results in activation of tyrosine kinase activity (Yarden *et al.*, 1987; Bishayee *et al.*, 1989; Honegger *et al.*, 1990; Li and Stanley 1991; Ullrich and Schlessinger 1990; Heldin *et al.*, 1995). The mechanism by which ligand binding induces dimerization is not yet known but is thought to involve conformational change in the receptor. Recently, mAbs to the fourth Ig-like domain of c-Kit or deletion of this domain were shown to abolish receptor dimerization and thereby inhibit signal transduction (Blechman *et al.*, 1995). This suggests receptor dimerization involves intrinsic interaction between receptor molecules and it is possible that the forth Ig-like domain is important in this process. It is possible that other domains within the receptor could also play a role in stabilizing receptor homodimers.

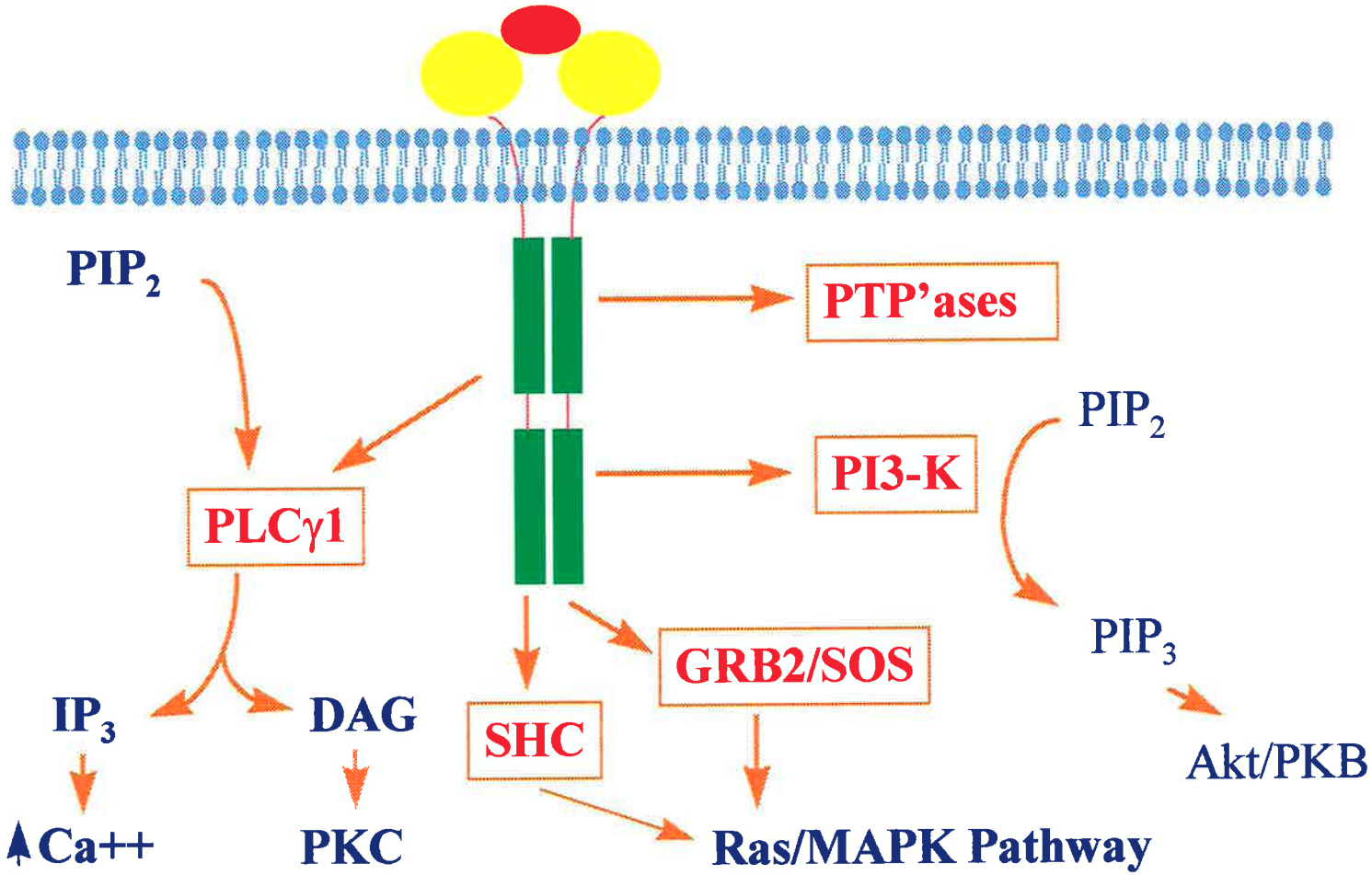
(B) Tyrosine kinase activity and signalling by c-Kit

It is known that Kit receptor homo-dimerization leads to phosphorylation of tyrosine residues within the receptor molecules via intrinsic catalytic activity (Hanks and Quinn 1991) which is an essential step in the initiation of signalling by the ligand-activated receptor. Systematic mapping of all the phosphorylation sites or substrate interaction sites has not been reported for c-Kit. A tyr residue in the kinase domain that has been shown to become phosphorylated upon c-Kit activation, is Y821 in murine c-Kit or Y823 in human c-Kit (Serve *et al.*, 1995). Mutation of Y821 to phenylalanine did not affect activation of PI-3K, p21ras, or MAPK or induction of the early response genes c-fos and c-jun. This mutation was unable to effect adhesion of BM derived mast cells (BMMC) to fibronectin which is normally induced by SCF,

however resulted in decreased proliferation (Serve *et al.,* 1995). This suggested that phosphorylation of this residue may only be important in some of the signalling pathways initiated by activation of Kit (Serve *et al.,* 1995). An overview of RTK substrates is shown in Figure 1.4. PI-3K is a heterodimer composed of an SH-2 containing regulatory unit of 85kDa (p85) and a catalytic unit of 110kDa. Activated c-Kit has been shown to bind and phosphorylate the p85 subunit in many different cell types (Lev *et al.,* 1991,1992a; Rieth *et al.,* 1991a, Rottapel *et al.,* 1991, Shearman *et al.,* 1993, Blume-Jensen *et al.,* 1994, Serve *et al.,* 1994), with Y719 of murine c-Kit or Y721 of human Kit in the interkinase region being the critical residues involved in the association. Mutation of this residue, besides decreasing PI-3K activation, also resulted in decreased induction of c-fos and c-jun, and inhibition of adhesion of BMMC to fibronectin. Together, these experiments suggest that the PI-3K pathway activated by c-Kit is involved in the induction of c-fos and c-jun and provides the signals necessary for adhesion but not proliferation in mast cells.

Activated c-Kit is also known to bind and phosphorylate Phospholipase C-γ1 (PLC-γ1)(Rottapel *et al.,* 1991, Lev *et al.,* 1991, Blume-Jensen *et al.,* 1994) and to bind to ras-GTPase activating protein (GAP)(Herbst *et al.,* 1991). PLC-γ1 is known to initiate the release of intracellular Ca^{2+} and activate Protein Kinase C (PKC) (reviewed in Cantley *et al.,* 1991). Recently, it has been reported that PI-3K and PLC-γ1 compete for association to their binding sites on the c-Kit receptor (Herbst *et al.,* 1995a). Other substrates that can bind directly to c-Kit are Tec kinase (Tang *et al.,* 1994), $p95^{vav}$ (Alai *et al.,* 1992), Grb-2 (Blume-Jensen *et al.,* 1994), JAK-2 (Weiler *et al.,* 1996) and c-Cbl (Wisniewski *et al.,* 1996). Other substrates that are indirectly activated in the signalling cascade include Ras, Raf-1 kinase and MAP kinase. Raf-1 can be tyrosine or serine phosphorylated and MAP kinase can be threonine or tyrosine

Figure 1.4: The signalling events following activation of c-Kit by SCF



phosphorylated (Lev *et al.,* 1991; Funasaka *et al.,* 1992; Hallek *et al.,* 1992; Okuda *et al.,* 1992; Welham and Schrader 1992, Blume-Jensen *et al.,* 1994). Raf-1 itself is also able to activate the MAP kinase pathway that results in the activation of specific transcription factors (reviewed in Schlessinger 1995). The exact signalling pathways that lead to specific cellular functions is not yet understood. It is however quite obvious that the signalling pathways initiated by c-Kit phosphorylation are very complex and the outcome of Kit activity may depend on the balance of the various pathways, the ratio of the different substrates and the specific cell type.

(C) Regulation of Kit activity

One known mechanism of regulation for all proteins occurs at the transcriptional level, where a decrease in gene expression leads to decrease of receptor levels. This can be induced by other cellular factors and does not seem to be dependent on Kit activity. IL-3 and GM-CSF have been shown to down-regulate c-Kit mRNA and protein in murine BMMC (Welham and Schrader 1991). Similarly TGF-β in CD34+ cells (Sansilvestri *et al.,* 1995), erythroid differentiation factor in murine erythroleukaemia cells (Hino *et al.,* 1995) and IL-4 in mast cell line HMC-1 as well as in AML cells (Sillaber *et al.,* 1991) have been shown to have the same effect.

Following activation of c-Kit by SCF, there are two other known mechanisms by which c-Kit activity is regulated; inhibition of tyrosine phosphorylation and down-modulation of the receptor. PKC, which can be activated by PLC-γ1, is able to induce serine phosphorylation of the c-Kit receptor, which inhibits the tyrosine phosphorylation of Kit thereby decreasing its activity (Blume-Jensen *et al.,* 1994). PKC is however not altogether an inhibitor of Kit function, since its activation is required for Kit induced cell motility (chemotaxis and migration) but not for proliferation (Blume-Jensen *et al.,* 1994). c-Kit activity is also negatively regulated by

phosphatases. In haemopoietic cells, the Haemopoietic cell phosphatase (HCP) also known as PTP1C, SHP1, SHPTP1, has been shown to associate with Kit and decrease its activity (Yi and Ihle, 1993; Paulson *et al.,* 1996; Piao *et al.,* 1996).

The other mechanism of negative regulation is initiated by the ligand/receptor complex itself, namely down-modulation due to receptor internalization. Following SCF binding, the Kit/SCF complex is internalized and targetted to the lysosomes for degradation or is ubiquitinated, also a trigger for protein degradation. Internalization of the soluble SCF and Kit receptor complex in mast cells and in the megakaryocytic cell line, MO7E (Yee *et al.,* 1993, 1994; Miyazawa *et al.,* 1994; Adachi *et al.,* 1995), results in decreased levels of cell surface Kit protein. Kit mutants have been used to demonstrate that Kit internalization / degradation does not occur in the absence of kinase activity by the receptor, suggesting that this mechanism is limited to activated receptors. Ubiquitination, a process that results in degradation, is also dependent on kinase activity (Yee *et al.,* 1994; Miyazawa *et al.,* 1994). Another process that leads to a dercrease in the levels of cell surface receptor is the shedding of the extracellular domain of the Kit receptor from the cell surface to give rise to a 100kD protein (Yee *et al.,* 1993). This mechanism is thought to be important in allowing the migration of cells where cell surface Kit is normally associated with membrane bound SCF (Yee *et al.,* 1993). This notion is supported by the fact that PKC, needed for cell motility, induces shedding of Kit receptor from the surface of murine myeloid cells (Brizzi *et al.,* 1994).

Soluble Kit receptors of 100kD have been detected in human serum and have been shown to be released by mast cells, human umbilical vein endothelial cells and leukaemic cell lines (Broudy *et al.,* 1994; Turner *et al.,* 1995, Wypych *et al.,* 1995). Native and recombinant soluble Kit molecules are able to bind SCF and have been

shown to act antagonistically to the activity of c-Kit surface receptors *in vitro*. It is possible that soluble c-Kit can bind to soluble or membrane bound SCF and inhibit its interactions with the cell surface c-Kit receptor (Langley *et al.,* 1993). Alternatively, soluble c-Kit could be able to dimerize with the extracellular portion of cell surface c-Kit receptors producing non-functional heterodimers, thereby inhibiting receptor activation by ligand.

Therefore as with most growth factor receptors, modulation of Kit receptor levels and regulation of its activity is complex and probably contributes greatly to the variety of its attributed functions. This probably reflects the requirements in a constantly modulating haemopoietic environment.

1.5 FUNCTION OF C-KIT AND ITS ROLE IN NEOPLASIA

1.5.1 The normal functions of c-Kit

The W and Sl mouse models (see section 1.4.4) implicate Kit/SCF in melanocyte function, germ cell development and in haemopoiesis (reviewed in Galli *et al.,* 1994; Lyman and Williams 1992).

Haemopoiesis is the process whereby mature myeloid and lymphoid cells derived from the pluripotent stem cells in a hierarchial manner. The stem cell present in the bone marrow has been shown to be able to repopulate the haemopoietic system of an irradiated recipient in most animal systems. This is due to its ability to expand and mature into multipotential progenitors that in turn give rise to lineage restricted / committed progenitors, that can differentiate into mature functional cells in the bloodstream and tissues. This process is dynamic and reflects a balanced response to competing stimulatory or suppressing influences.

The Kit/SCF interaction seems to play an important role in maintaining a bone marrow environment necessary for haemopoiesis. Only a fraction of total murine (8%) and human (4%) BM cells express c-Kit, with the stem cells and early progenitors being positive for surface expression (Ashman *et al.,* 1991; Ogawa *et al.,* 1991; Okada *et al.,* 1991; Brandt *et al.,* 1992). As early progenitors differentiate and become lineage comitted, the expression of c-Kit is reduced (Ogawa *et al.,* 1991); however it is found to be expressed on mature mast cells (Mayrhofer *et al.,* 1987) and megakaryocytes (Avraham *et al.,* 1992a,b). In both these mature cell types SCF is able to enhance proliferation and maturation (Nocka *et al.,* 1990a; Williams *et al.,* 1990; Grossi *et al.,* 1995; Kiss *et al.,* 1996). In the murine and human systems, SCF activity in *in vitro* experiments has been demonstrated to have mostly a synergistic effect with other cytokines including GM-CSF, G-CSF, Epo, IL-3, IL-4, IL-6, IL-7, IL-9 and IFN-γ (Broxmeyer *et al.,* 1991a,c; Carow *et al.,* 1991; Hoffman *et al.,* 1993). Kit also plays a role in chemotaxis of haemopoietic progenitor cells and has been shown to induce a migratory response (Okumura *et al.,* 1996). In two different studies c-Kit has also been shown to promote cell survival by suppressing p53 induced apoptosis (Abrahamson *et al.,* 1995) and, in natural killer cells, SCF was found to upregulate bcl-2 also leading to a suppression of apoptosis (Carson *et al.,* 1994). However, in metastatic melanoma cells, enforced c-Kit expression caused these cells to become apoptotic in response to SCF (Huang *et al.,* 1996). This and other evidence suggests that the actions of Kit may vary depending on the cell type, the presence of other cytokines, concentration of ligand and even levels of the Kit receptor itself.

1.5.2 Kit in neoplasia

c-Kit not only plays a role in haemopoiesis and in embryogenesis but, like other proto-oncogenes, is associated with malignancy. Many members of the RTK 3

family have been linked to tumourigenicity and display oncogenic potential (reviewed in Ullrich and Schlessinger 1990). The first evidence of oncogenic Kit was with its discovery via the v-*kit* oncogene (see section 1.4.1) which encoded a terminally truncated, constitutively active form of Kit (Besmer *et al.,* 1986). Independently, c-Kit was identified at the protein level as a marker in a subset of patients with human acute myeloid leukaemia (Gadd and Ashman, 1985) and high level of expression was shown to be associated with poor prognosis (Ashman *et al.,* 1988). Since then it has been demonstrated that introduction of a c-*kit* expression construct into NIH-3T3 fibroblasts could cause transformation (Alexander *et al.,* 1991). Due to the expression of c-Kit in specific cell lineages of the haemopoietic system, as well as on specific non-haemopoietic tissues (see section 1.4.3), the involvement of Kit with haemopoietic disorders or leukaemias, mast cell malignancies, melanoma, gonadal and breast tumours, brain tumours and lung cancer has been investigated by various researchers.

Even though c-Kit plays a role in gametogenesis and is expressed in germ cells, there has been no evidence of amplification or gross re-arrangement of the kit gene in any of the gonadal tumours examined, but high levels of transcripts were observed in a large percentage of human seminomas* (reviewed in Galli *et al.,* 1994). In an investigation using an *in vivo* model of papillomavirus-induced tumourigenesis, it was shown that c-Kit activation via SCF, was essential for testicular tumourigenesis (Kondoh *et al.,* 1995). In another study it was suggested that Kit does play a role in oncogenesis of germ cell tumours, where down-regulation of c-*kit* could be important in progression from seminomas to non-seminomas (Izquierdo *et al.,* 1995). From various studies it was concluded that there was no alteration of Kit expression or activity in brain tumours, neuroblastomas or breast cancers even though c-Kit plays a

* see additional notes

role in neural tissue development and the normal development and function of mammary cells (reviewed in Galli *et al.,* 1994; Beck *et al.,* 1995). However, some cases of small cell lung cancer, neural tumours and breast cancers have been found to express both Kit and SCF (Hines *et al.,* 1991; Turner *et al.,* 1992; Matsuda *et al.,* 1993; Cohen *et al.,* 1994; Ryan *et al.,* 1994; Stanulla *et al.,* 1995; Hines *et al.,* 1995; Krystal *et al.,* 1996). It is possible that stimulation by autocrine SCF may be important in these cancers, thus providing a link between Kit/SCF and malignant solid tumours. In lung cancer, especially, most studies have found that small cell lung carcinoma (SCLC) cells also co-express Kit and SCF, and in culture were shown to sercete SCF into the medium. There have also been several reports to indicate that the expression of c-Kit is increased in SCLC (Sekido *et al.,* 1991; Hibi *et al.,* 1991; Matsuda *et al.,* 1993). Hence it has been postulated that an autocrine or paracrine growth loop of SCF and Kit signalling may be important in the continuous proliferation/transformation of SCLC cells (Matsuda *et al.,* 1993; reviewed in Galli *et al.,* 1994).

In earlier studies, the level of Kit was found to be reduced in malignant melanocytes compared to normal cells and it was suggested that Kit provided a differentiative effect in these cells (Natali *et al.,* 1992). In another study, metastatic melanoma cells were found to be heterogeneous in the level of Kit expressed and SCF was found to be unable to induce proliferation in these populations (Funasaka *et al.,* 1992). However, over-expression of Kit (in the melanoma cells that did not express any endogenous c-Kit) caused these cells to become apoptotic when expossed to SCF (Funasaka *et al.,* 1992). Hence, in melanoma the lack of Kit expression or response to SCF may be important in allowing an escape from cell death (Huang *et al.,* 1996).

Recently, most of the research on Kit in malignancies, has focussed on disorders of the haemopoietic system, in particular, mast cell leukaemias. c-Kit mRNA and protein are expressed in blast cells from AML but not ALL patients (Gadd and Ashman 1985; Ashman *et al.,* 1988; Wang *et al.,* 1989; Lerner *et al.,* 1991). However, c-Kit is expressed by some lymphoid progenitors and functional c-Kit was demonstrated in a T cell ALL line (Kees and Ashman, 1995). Studies have demonstrated a proliferative response by some AML blast cells to SCF alone *in vitro* (Ikeda *et al.,* 1991; Wang *et al.,* 1991; Broudy *et al.,* 1992; Carlesso *et al.,* 1992) as well as in synergy with GM-CSF, G-CSF and/or IL-3 (Ikeda *et al.,* 1991; Wang *et al.,* 1991; Broudy *et al.,* 1992; Carlesso *et al.,* 1992, Goselink *et al.,* 1992). SCF can also act in synergy with IL-3 or GM-CSF to induce proliferation of CML blast cells in vitro (Goselink *et al.,* 1992). Over-expression of certain RTKs notably HER2/neu has been associated with oncogenesis (Slamon *et al.,* 1987; Slamon *et al.,* 1989) and in one study (as mentioned previously) high level expression of c-Kit was associated with poor prognosis in AML (Ashman *et al.,* 1988), although this was not found in other studies (Wang *et al.,* 1989; Reuss-Borst *et al.,* 1994). Comparison with normal CD34+ BM cells showed that c-Kit protein is rarely over-expressed in AML and gene amplification was not observed (Cole *et al.,* 1996). Furthermore, autocrine production of SCF could not be detected in AML (Cole *et al.,* 1996). In another recent study, the level of expression of c-Kit did not to correlate with any significant difference in remission rate or survival in acute leukaemia (Lauria *et al.,* 1995). Conversely, the level of soluble c-Kit in serum was found to be elevated in a marked number of AML and CML patients but not in ALL patients, and the level of soluble c-Kit decreased to normal after effective chemotherapy (Kawakita *et al.,* 1995).

In patients with myeloproliferative disorders, a point mutation in codon 52 resulting in an Asp to Asn change in the extracellular domain of c-Kit has been detected, but its effects on Kit activity or function is not yet known (Nakata *et al.,* 1995). A number of activating mutations have been linked to mast cell malignancies in the mouse, rat and human. Among blast cells from patients with different leukaemias, a point mutation was found in the tyrosine kinase domain of Kit in all four patients in the group showing 'mastocytosis with an associated haematological disorder' (Nagata *et al.,* 1995). This identical mutation causing a Asp to Val amino acid substitution at residue 816, was previously detected in a human mast cell line HMC-1, and shown to result in constitutive phosphorylation of Kit in the absence of ligand (Furitsu *et al.,* 1993). The corresponding mutation occurs also in other species and has been detected in the P815 murine mastocytoma cell line, as an Asp814 to Tyr mutation (Tsujimura *et al.,* 1994), and in the rat RBL-2H3 mast cell line as Asp817 to Tyr mutation (Tsujimura *et al.,* 1995). This mutation and the Val559 to Gly mutation in the juxtamembrane domain, also initially indentified in HMC-1, both result in constitutive Kit phosphorylation in the human embyonic kidney cell line 293T and in the murine myelomonocytic factor-dependent cell line FDP-C1 (Tsujimura *et al.,* 1994; Kitayama *et al.,* 1995). In FDP-C1 cells these muatnts are also able to confer factor independent growth and tumourigenic potential in nude mice (Kitayama *et al.,* 1995). In the IC-2 murine factor dependent mast cell line, these mutants not only induced factor-independence and tumourigenicity but also induced differentiation to mature mast cells (Hashimoto *et al.,* 1996). However, introduction of this mutant form of Kit into the BM cells of mice resulted in the growth of granulocyte-macrophage, mast and mixed colonies *in vitro,* but resulted in immature acute lymphoblastic leukaemias *in vivo* (Kitayama *et al.,* 1996). Another deletion of 7 amino acids in the

juxtamembrane region of c-Kit has been detected in a murine mastocytoma cell line FMA-3 (Tsujimura *et al.,* 1996). This mutation may work in a similar manner to the mutation (G559) in the same region found in HMC-1 cells, which is thought to function by inducing constitutive dimerization (Kitayama *et al.,* 1995), thereby leading to phosphorylation and constitutive activation.

The most common mutation V816Kit not only causes constitutive activation of the Kit receptor but has recently been shown to alter the substrate specificity of Kit and cause degradation of (a known negative regulator of Kit activity) Haemopoietic Cell Phosphatase (HCP) by the ubiquitin-dependent proteolytic pathway (Piao *et al.,* 1996). HCP is known by many other names including SHP-1 and SHPTP, and is also a negative regulator of signalling by other cytokine and growth factor receptors. This opens up many new possibilities for the action of mutant oncogenes, especially variation in their normal function and possible alterations in the regulation of other cellular proteins that are not normally directly involved with their activity.

1.6 SYNERGY BETWEEN ONCOGENES

It was previously believed that certain oncogenic proteins like mutant Ras were able to cause transformation alone, in what seemed to be a one step process. However, it is now accepted that cancer occurs in several stages and is indeed a multi-step process (reviewed in Land *et al.,* 1983b; Bishop 1991; Varmus 1989; Hunter 1991). Early studies showed that DNA tumour viruses carried multiple transforming genes encoding proteins that acted co-operatively e.g. the large and middle T antigens of polyomaviruses (Rassoulzadegan *et al.,* 1982) or the products of E1A and E1B genes of adenoviruses (Levine, 1984). Addditionally, some retroviruses were also found to carry more that one oncogene; for example AEV carries both *erbA* and *erbB*

genes (Hayman and Beug, 1992) and E26 carries a *myb/ets*-1 fusion gene (Nunn *et al.*, 1984). These observations suggested that oncogene co-operation was important in transformation. Gene transfer studies proved that although primary embyronic fibroblasts could not be easily transformed by a single oncogene, combinations of oncogenes proved successful (Land *et al.*, 1983a). Transfection studies were used by many to reveal oncogenes that were able to co-operatively cause transformation (reviewed in Weinberg 1989; Ruley 1990; Hunter 1991; Land *et al.*, 1983b). Many naturally occuring tumours were found to contain multiple mutations (reviewed in Ruley 1990; Hunter 1991; Land *et al.*, 1983b). Furthermore, many oncogenes were found to act co-operatively in various types of lymphomas and leukaemias, for example mutant *myc* was associated with p53 in many lymphomas, with *bcr-abl* in CML and mutant p53 or *ras* in several other types of leukaemia (reviewed in Adams and Cory 1992). These are only some of many malignancies now documented in which oncogenes acting co-operatively have been defined. It is now evident from these studies, that specific events (listed below) which involve mutations in particular types of oncogenes that act co-operatively to induce transformation, lead to haemopoietic neoplasia. (1) The mutation of an oncogene that enhances the lifespan of the cell, for example activation of an 'immortalising' agent like *myc* or *myb*. (2) A mutation that results in 'factor-independence' for example autocrine production of a growth factors like CSF-1 or IL-3 or constitutive activation of a cytokine receptor or signalling protein like *ras* or *raf* (Wienberg, 1985; reviewed in Weinberg, 1989; Adams and Cory, 1992). (3) The mutation of a tumour suppressor gene like p53 or Rb (reviewed in Weinberg, 1991). Since cancer is a multi-step process, it is now accepted that two or more of the above events can act co-operatively to result in cellular transformation.

1.7 AIMS

The two oncogenes of interest in this study are those described in detail above: the nuclear transcription factor Myb and the receptor tyrosine kinase Kit. The aims of this study involved the assessment of the effects of over-expression of each individually and in co-operation, in murine haemopoietic cells. As mentioned previously, *myb* oncogenes carried by avian retroviruses and activated forms of murine c-*myb*, all contain terminal truncations. It has already been shown that structurally altered activated forms of Myb can result in factor-dependent transformation of primary haemopoietic cells derived from foetal liver. However, it was not clear whether structural alteration of Myb was necessary for its oncogenic capacity. The first aim of this study was to investigate the effects of over-expression of full length c-Myb in murine primary FLC, in comparison to activated C-terminally truncated Myb.

Myb infected long term cell lines are continuously proliferating, displaying features of immature progenitor cells, but are dependent on exogenous growth factor for survival (reviewed in Gonda 1991; Gonda *et al.,* 1993). These cell lines, derived by introduction of C-terminally truncated Myb into primary haemopoietic cells, are not tumourigenic but have been shown to become tumourigenic after acquiring factor-independence (Gonda *et al.,* 1989b). Other studies that will be described in more detail in following chapters suggest that activation of *myb* is an early step in transformation, and that additional co-operative oncogenes are necessary for the development of any haemopoietic disorders. This prompted the investigation of the effects of activated Myb in an *in vivo* murine model, with the second aim being to determine if activated Myb expressed in the haemopoietic system of mice is able to result in any form of malignancy.

A possible candidate for an oncogene able to co-operate with Myb is Kit. These two candidates also satisfy the known properties of co-operative oncogenes by their complementary modes of action, Myb being an 'immortalising' agent and activated Kit able to confer factor-independence. To determine the co-operative effects of the two oncogenes in primary haemopoietic cells it was of importance to determine the effects of each individually. As the effects of activated Myb in FLC are already known, the third aim was to observe the effects of over-expression of activated Kit individually, and in co-operation with activated Myb in primary FLC.

Activated forms of Kit have been shown to confer factor-independence in myelomonocytic FDC-P1 cells (which require GM-CSF or IL-3 for survival) as well as in mast-like IC-2 cells which also exhibited a differentiation response. To specifically analyse the effects of activated Kit, long term 'Myb-transformed' myeloid cell lines were chosen as an ideal model system, as these cells were factor-dependent and had also previously been shown to differentiate in response to specific factors. Use of these cells would also enable further assessment of oncogene co-operation between activated Myb and activated Kit.

To investigate the effects of over-expression of full length c-Myb in murine primary FLC, activated C-terminally truncated CT3Myb and WTMyb were compared in transformation assays, and the cell lines obtained were analysed phenotypically and morphologically.

To determine the effects of constitutive expression of activated Myb in the haemopoietic cells of mice over a long term period we used a BM transplantation system in order to assess any malignancies induced by mutated Myb.

To analyse the effects of constitutively active Kit in primary haemopoietic cells, a mutant form of human Kit (V816) was introduced into FLC, and the cell lineages

obtained, as well as the clonogenic potential was assessed in the absence of factor or in the presence of human SCF.

To observe the synergetic effects of activated Myb and Kit in myeloid cells, activated human Kit or normal human c-Kit was introduced into long term factor-dependent myeloid haemopoietic cell lines (expressing activated Myb) and the effects of withdrawal of factor or the presence of human SCF on the phenotypic and fuctional characteristics were analysed.

Chapter 2. MATERIALS AND METHODS

2.1 TISSUE CULTURE

2.1.1 Tissuse culture Media, Solutions and Cytokines

Dulbecco's Modified Eagle's Medium (DMEM) was prepared by mixing one sachet of DMEM powder (Gibco, USA, Cat. No. 12800-017) and 3.7g of $NaHCO_3$ (BDH, USA, Cat. No. 10247) in 900ml of Milli-Q purified water (MQ-water) (tap-water deionised using a Milli-Q RO60 system and purified through two beds of ion exchange resins, a carbon filter and an organic filter of a Milli-Q system - Millipore, USA). To the dissolved solution, N-2-Hydroxyethylpiperazine N`-2-ethanesulphonic acid (HEPES) pH 7.2 (Boehringer-Mannheim, Australia, Cat. No. 737151) was added from sterile stock solutions to give a final concentration of 15mM. Likewise, penicillin (Sigma, USA, Cat. No. P3032) and streptomycin sulphate (Sigma, USA, Cat. No. S9137) were added to final concentrations of 100IU/ml and 100µg/ml. The pH was adjusted to 7 by adding 4ml of 1M Hydrochloric Acid (HCl) and the volume adjusted to 1L using MQ-water.The medium was filter sterilised using an Acrocap™ 0.22µm filter unit (Gelman Sciences, Michigan, USA, Cat. No. 4480) and a Millipore pump with a filling bell (Millipore, USA, Cat. No. SVGB1010) and stored at 4°C.

Iscove's Modified Dulbecco's Medium (IMDM) was prepared by adding one sachet of IMDM powder (Gibco, Cat. No. 12200-036) and 2g of $NaHCO_3$ in 900ml of MQ-water. As with DMEM, sterile stock solutions of HEPES, penicillin and streptomycin sulphate were added to the same final concentrations as indicated above and filter sterilised as indicated above. The pH was adjusted to 7.4 with 2.5ml of HCl and the volume to 1L. RPMI 1640 medium was prepared the same way as for IMDM using RPMI 1640 powder (Gibco, Cat.No. 31800-02).

Double strength (ds) IMDM was prepared by dissolving one sachet of IMDM powder (Cytosystems, Australia, Cat. No. 50-016-PA) and 0.2g L-asparagine in 390ml of MQ-water. Sterile stocks of penicillin, streptomycin, DEAE-Dextran (Pharmacia, Sweden, Cat. No. 17-0350-01) were added to give final concentrations of 200 IU/ml, 200μg/ml and 0.19mg/ml respectively. Medium was filter-sterilised and stored up to 6 months at 4°C.

Prior to use all media were supplemented with 10-20 % v/v foetal calf serum (FSC) (Gibco, USA, Cat. No. 200-6140PJ, Batch No. 660; heat inactivated at 56°C for 30 mins) as well as glutamine (BDH, Cat. No. 37107) to a final concentration of 2mM and warmed to 37°C. Fresh glutamine (2mM final) was added to medium stored at 4°C for more than 7 days.

Analytical grade reagents were used in the preparation of all solutions. Solutions used to wash cells were Hank's balanced salt solution (HBBS) and Tissue Culture Phosphate Buffered Saline (TC-PBS). HBBS contained 0.14M NaCl, 5mM KCl, 0.3mM $Na_2HPO_4.12H_2O$, 0.4mM KH_2PO_4, 4.2mM $NaHCO_3$, 5.5mM glucose and 1% phenol red (M & B, England, Cat. No. P152/18/61) dissolved in 0.1M NaOH in MQ-water with a final pH of 7.4. TC-PBS contained 0.14M NaCl, 3mM KCl, 8mM $Na_2HPO_4.12H_2O$ and 1mM KH_2PO_4 in MQ-water with a final pH of 7.4. The solutions were sterilised by autoclaving at 130°C for 20 mins.

A solution of 0.054% w/v trypsin (Difco, USA, Cat. No. 0152-13-1) and 0.54mM Ethylenediaminetetra-acetic acid (EDTA) in HBBS, purified through a low protein binding 0.22μm filter (Millipore, USA, Cat. No. SLGV025LS) and stored at -20°C, was used to remove adherent cells from tissue culture dishes or flasks.

Semi-solid medium using a methylcellulose base was used for growing colonies. To a 500ml bottle, prior to autoclaving, 8.1g methylcellulose powder (DOW

Chemical Co., A4M premium grade) and a magnetic flea was added. When cooled, 270ml of sterile single strength IMDM (double-strength diluted 1:2) was added while stirring and the mixture left stiring for 3days at room temperature in the dark. During this time, a fresh batch of Bovine Serum Albumin (BSA) solution was made by dissolving 20g of BSA (Sigma, USA, Cat. No. 2153) in 88.4ml MQ-water in a conical flask at 4^0C overnight. The solution was deionised with Duolite mixed resin beads (BDH, Aust., Cat. No. 55057) at 4^0C. This involved adding 4g of beads to the BSA solution for 4 hours with mixing every 15 minutes followed by filtration to remove the beads, with the process being repeated twice more. The BSA solution was decanted, an equal volume of double-strength IMDM was added, and filter sterilised. To the disolved methylcellulose, 60ml of this BSA solution and 180ml of FCS was added and left stirring for a further 4 hours. The methylcellulose mixture was aliquoted and stored at -20^0C until required.

Cytokine units are defined such that 50 Units results in 50% of the maximum number of colonies in soft agar cultures containing $5x10^4$ murine BM cells. In all experiments either murine cell lines or primary cells were used, hence most cytokines were murine except where indicated. Recombinant granulocyte macrophage - colony stimulating factor (GM-CSF) at 40,000 U/ml, synthesised by yeast expressing a GM-CSF expression vector, was a gift from Drs. T. Wilson and N. Gough (Walter and Eliza Hall Institute, Melbourne, Aust.) and recombinant interleukin-3 (IL-3) at $8.3x10^5$ U/ml, synthesized by insect cells infected with a recombinant baculovirus vector was a gift from Dr. A. Hapel (John Curtin School of Medical Research, Canberra, Aust.). Purified recombinant murine Stem Cell Factor (SCF) was a gift from Dr. D. Williams and collegues (Immunex Corp., Seattle, WA) and Human SCF

produced in E.*coli*, was supplied by Amgen Corporation (Thousand Oaks, CA). Human interleukin-6 (IL-6) was obtained from Paul Simmons (HCCR, Adelaide).

2.1.2 Cell lines and culture maintenance

All cells were maintained in tissue culture flasks (Corning, USA. Cat.No. 25110-25) containing tissue culture medium and incubated at 37^{0}C in a humidified atmosphere of 5% CO_2 in air. Cells were either subcultured or the tissue culture medium was replaced every 3 to 5 days depending of the growth rate. All suspension cell cultures were maintained for a maximum of 3 months and adherent cultures for a maximum of 6 weeks, after which cultures were re-establised from cryopreserved stocks. Cell densities were calculated using a haemocytometer and exclusion of trypan blue (0.8% w/v in saline) diluted 1:2 was used to determine cell viabilities.

The murine fibroblast cell lines, NIH-3T3 and Ψ2 (Mann *et al.*, 1983) were maintained in DMEM / 10% FCS as adherent monolayers. Confluent or near-confluent cell monolayers were harvested by rinsing in HBBS and then incubating at 37^{0}C for 1min with trypsin-EDTA-HBBS solution. The trypsin was inactivated by the addition of DMEM / FCS and flasks were re-seeded with $2x10^5$-10^6 cells. The murine factor-dependent cell line, FDC-P1 (Dexter *et al.*, 1980) was cultured in DMEM / 10% FCS supplemented with 80 U/ml GM-CSF with cultures initiated at 10^4-$5x10^4$ cells/ml.

All primary cells were derived from fresh murine tissues. FLC were maintained in DMEM / 20%FCS and BM cells in IMDM / 20%FCS with the addition of relevant cytokines. GM-CSF was used at a final concentration of 1000 U/ml, IL-3 at 500 U/ml, SCF at 100ng/ml, and IL-6 at 10ng/ml in all primary cell cultures. All non-adherent primary cells were washed in HBBS / 2%FCS.

2.1.3 Cryopreservation and thawing of cells

For storage, cells in culture were harvested at log phase, washed in HBBS and resuspended at $5x10^6$ - $2x10^7$ cells/ml in medium / 10%FCS. Dropwise an equal volume of cryoprotectant (30% heat-inactivated FCS, 20% dimethyl sulphoxide (DMSO) (BDH, Merck,Vic., Aust. Cat. No. 10323) and 50% RPMI) was added. 1ml aliquots of the cell suspension in cryotubes (Nunc, Denmark, Cat No. 3-66656) were cooled to -100^{0}C in liquid nitrogen vapour for 3 hours - 1 day and then transferred to into liquid nitrogen for long term storage.

For culuring cryopreserved cells, cryotubes from liquid nitrogen were rapidly thawed at 37^{0}C in a water-bath. The cell suspension was transferred to a 10 ml tube and an equal volume of medium/ 10%FCS (warmed to 37^{0}C) was added dropwise, mixed and allowed to stand for 5 mins. The suspension was further diluted in medium./10%FCS to 10ml and centrifuged at 200g for 5mins. The cells were washed in HBBS and cultured as usual.

2.2 CYTOLOGY, CYTOCHEMISTRY AND HISTOLOGY

2.2.1 Preparation of cell smears and tissue samples

Cells were harvested from culture, resuspended in medium containing over 50% FCS and 2 x 10^4 - 10^5 cells were deposited onto microscope slides by centrifugation at 5000rpm for 5 mins in a Cytospin 3 centrifuge (Shandon Scientific Ltd., England). Slides were allowed to air dry and stained within 48hrs or stored at 4^{0}C in air tight boxes in the presence of self-indicating 2- 4 mm silica gel (Ajax Chemicals, Aust. Cat. No. 3681) for up to a maiximun of 10 months and warmed to room temperature within the sealed boxes immediately prior to use.

To observe cellular morphology, tissue samples and tumor specimens were fixed in formalin (40% v/v formaldehyde in distilled water). The specimen samples were then paraffin embedded, sectioned at 5μm by the Division of Histopathology, IMVS. The sections were stored at room temperature and stained as described below.

To detect specific markers, tissue samples or tumor specimens were placed in Tissue TeK OCT compound (Miles Inc. IL), frozen by immersion in isopentane pre-cooled in liquid nitrogen and stored at -70°C. Sections (5μm thick) from the fresh frozen samples were prepared by the Division of Histopathology, IMVS, and stored similarly to the cell smears at 4^0C as detailed above.

2.2.2 Morphological characterisation of cells

To observe general cellular morphology, cell smears were stained with Wright-Giemsa through the automated system by the Diagnostic Services Laboratory, Division of Haematology, IMVS. Briefly, the slides were immersed in Jerner's stain for 2 mins, Giemsa stain stain for 6 mins and washed in a buffer of pH 7, before being air-dried. The cells were mounted in DePX.

To observe the morphology of tissue samples, sections were stained with Haematoxylin/Eosin by the Division of Histopathology, IMVS and then mounted in DePX.

2.2.3 Phenotypic characterisation of cells

Enzyme stains to detect expression of lineage specific esterases were used to confirm the phenotype of cells derived from cell culture.

The method to detect the lineage specific enzymes "non-specific" (α-naphthyl acetate) esterase expressed by mature macrophages and chloro-acetate esterase expressed by mature neutrophils was adapted from that described by Yam *et al.*, (1971). Cytocentrifuged cell smears were fixed in esterase fixative (see appendix 1.1)

for 30sec at 4°C and washed 3 times in distilled-water. In a Coplin jar slides were allowed to stain for 45 mins at room temperature in a freshly made solution of 'non-specific' esterase substrate solution (see appendix 1.1). The slides were washed 3 times in distilled-water and stained for 1 hour at room temperature in a freshly made 'chloroacetate' esterase substrate solution (see appendix 1.1). The slides were again washed 3 times in distilled water and counterstained in a methylgreen solution (see appendix 1.1). Cells expressing 'non-specific' (α-naphthyl acetate) esterase were detected by a red-brown colouring and those expressing 'chloroacetate' (napthol-AS-D-chloroacetate) esterase by a blue colouring. For photography slides were mounted in glycerol/glycine mountant (see appendix 1.1).

The following method was used to detect acetylcholinesterase expressed by murine megakaryocytes and is essentially similar to that described by Karnovsky and Roots (1964). Cells were fixed in cold esterase fixative (see above) for 30 sec and washed 3 times in distilled water. The slides were rinsed in 0.1M phosphate buffer pH 6 (see appendix 1.1) and stained in an fresh acetylthiocholine solution (1.5ml 0.1M sodium citrate, 3ml 30mM copper sulphate and 3ml 5mM pottassium ferricyanide was added to 22.5 ml 0.1M phosphate buffer pH 6 sequentially while stiring) for 4 hrs at room temperature in the dark. The slides were washed 3 times in distilled water, counterstained in Haematoxylin and mounted in glycerol/glycine mountant (see above).

2.3 IMMUNOASSAYS

2.3.1 Antibodies

Antibodies against surface markers were used for immunofluorescence and immunohistochemistry. Mouse monoclonal 1DC3 raised against human c-Kit (Aylett

et al., 1995) was produced in our laboratory and used to detect expression of huKit. Rat monoclonal antibodies that recognize specific murine lineage surface markers were used in phenotypic charaterisation of cells. These included: 30H12 raised against Thy1 (Ledbetter and Herzenberg, 1979) (obtained from Dr. I. Kotlarski, University of Adelaide); 8C5 raised against Gr-1 (Holmes *et al.*, 1986) and M1/70 raised against Mac-1 (Springer *et al.*, 1979) (donated dy Dr. I. Bertoncello, Peter Macallum Institute, Melbourne); ACK-2 raised against murine c-Kit (Ogawa *et al.*, 1991) (donated by Dr. M. Ogawa, Dept. Of Pathology, Institute for medical Immunology, Kumamoto University Medical School, Japan); F4/80 raised against mature macrophages (Austyn and Gordon, 1981) (donated by Dr. A. Hapel, John Curtin School of Medical Research, Canberra). Mouse monoclonal antibodies raised against different congenic mouse strain surface markers of CD45 (Morse, 1992) including A20.1 (anti-Ly5.1, now also known as $Ly5^a$: the CD45.1 antigen) and ALI-4A2 (anti-Ly5.2, also called $Ly5^b$: the CD45.2 antigen) were both donated by Dr. C. Li, Queensland Institute of Medical Research and used in the analysis of the transplantation assay (section 2.11).

To detect primary monoclonal antibodies affinity-isolated fluorescein isothiocyanate (FITC)-labelled $F(ab')_2$ sheep antibody to mouse immunoglobulin (Silenus, Australia, Cat.No. DDAF) or phycoerythrin (PE) labelled goat anti-mouse (Southern Biotechnology Associates, Inc. Birmingham, USA Cat. No. 1030-09) were used. To detect biotinylated antibodies, streptavidin R-PE (Catlag Laboratories, San Francisco, CA. Cat. No. SA 1004-4) was used at a dilution of 1:50 for immunofluoresence.

2.3.2 Immunofluorescence Assay and Fluorescence - activated cell sorting

Target cell suspensions from cultured cell lines were washed twice in PBS containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide (PBA). These

cells were then resuspended in 10^7 cells/ml in PBA supplemented with 10% heat inactivated normal rabbit serum (PBA/NRS) to block the binding of antibodies to Fc receptors on the cells. Aliquots of 100µl of cells plus antibody were incubated on ice for 1 hour. The cells were then washed 3 times in cold PBA and incubated with secondary antibody on ice in the dark for 45 mins. The cells were washed twice in cold PBA, fixed in 0.5 - 1 ml of cold PBS containing 1% paraformaldehyde (BDH, Cat. No. 29447) and stored for up to 1 week at 4^0C in the dark. The samples were analysed on a Profile II flow cytometer (Coulter, Hialeah, FL). To ensure reproducibility of the measured mean fluorescence intensity (MFI) on different days, the photomultiplier tube sensitivities were standardised using Standard-Brite™ calibration beads (Coulter, Hialeah, FL, Cat. No. PN 6604146).

For preparative sorting, cells were stained as above in the absence of sodium azide, under sterile conditions and resuspended in cold PBA. The cells were passed through a FACSSTAR^{plus} cell sorter (Becton-Dickinson, Mountainview, CA) and cells positive for specific marker expression were collected as indicated.

2.3.3 Immunocytochemistry

The Alkaline phosphatase anti-alkaline phosphatase (APAAP) method, derived from that described by Erber *et al.,* (1984), was used to detect specific cellular proteins. This procedure was performed at RT, except for incubation of primary antibody overnight as indicated. Cell smears were prepared from cultured cells (as outlined in Section 2.2.1). A circle was drawn around the cell smear using a Dakopen (Dakopatts, Denmark, Cat.No. S2002) to allow antibody solutions to be localised. The cells were fixed for 30 secs by immersion in 5% formaldehyde, 47.5% acetone and 47.5% methanol. The slides were rinsed in distilled water and washed 3 times in Tris-buffered saline (TBS) (see appendix 1.1). Primary antibody was diluted in PBS/10%

NRS and applied to the cell smear. The slides were incubated for 2 hrs at RT or overnight at 4°C in a humidified container. The sides were washed 3 times in TBS and secondary antibody, rabbit anti-mouse Ig (Dakopatts, Denmark, Cat. No. Z259) diluted 1:50 in 25% heat inactivated normal human serum (NHS) in TBS, was added for 30 mins. The slides were washed 3 times in TBS and incubated for 30 mins with tertiary antibody, alkaline phosphatase anti-alkaline phosphatase mouse monoclonal (APAAP) (Dakopatts, Denmark, Cat. No. D651) diluted 1:100 in TBS. The additions of secondary and tertiary antibodies were repeated twice more with each incubation for 10 mins followed by 3 washes in TBS. The slides were placed in substrate (20 mg napthol AS-MX phosphate (Sigma, USA, Cat.No. L9756) dissolved in 2 ml dimethylformamide (BDH, Aust. Cat. No. 10322) and made up to 100 ml with Tris-HCl pH8.2, to which 100µl of 1M levamisole (Sigma, USA) and 1mg/ml final of Fast Red (Sigma, USA, Cat. No. F1500) was added) at RT for 30 mins. They were washed and counterstained with Gill's Haematoxylin (a solution of 25% v/v Ethylene Glycol and 2% v/v glacial acetic acid in distilled water, containing 2g/L of anhydrous/monohydrate Haematoxylin, 0.2g/L sodium periodate ($NaIO_4$) and 17.6g/L aluminium sulphate ($Al_2(SO_4)_3.18H_2O$). Cells were washed and mounted in a glycerol / glycine mountant (30ml of 1.4% w/v glycine solution pH 8.6 and 70ml glycerol).

To detect murine mast cells a variation to the above technique was used. Cells were fixed as above and rabbit polyclonal antibody raised against a synthetic peptide of murine mast cell protease-5 (anti-mMCP-5), provided by Dr. Patrick M°Neil, University of New South Wales, Sydney (McNeil *et al.,* 1992), was used at a 1:25 dilution as primary antibody and incubated for 2 hours. Biotinylated swine anti-rabbit Ig (Dako, Carpinteria, CA) diluted 1:500 in PBS/20%NHS was used as secondary antibody, followed by incubation for 1 hour with neat strepavidin-Alkaline

Phosphatase (Zymed Laboratories, CA, USA, Cat. No. P50237). Cells positive for MCP-5 were detected using the substrate detailed above in the APAAP method.

2.4 RECOMBINANT DNA TECHNIQUES

2.4.1 Restriction Endonuclease digestion

Plasmid DNA at a concentration of up to 2 μg/μl was digested with a restriction endonuclease with (used at a 2-5 fold excess) according to the method recommended by the manufacturer (New England Biolabs, Amersham, Promega or Pharmacia) in a final volume of up to 50μl. Digests were incubated for 1 - 3 hours and most reactions were terminated by heating at 65°C for 10 minutes. Digestion buffers used were those specified by the manufacturers. Genomic DNA of 0.5- 2μg/μl was digested similarly, but for 10 - 20 hours in a final volume of up to 50μl.

2.4.2 Analysis and Quantitation of DNA

DNA samples were diluted in MQ-water with 10% loading buffer (Sambrook *et al.,* 1989) and agarose gels of 0.8%, 1% or 1.5% (w/v) in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA), depending on the size of the DNA fragments to be fractionated. DNA samples in agarose gels were electrophoresed in a horizontal gel apparatus containing 1 x TAE buffer at 100 V for 2 - 3 hours. The gels were stained in 2μ g/ml ethidium bromide (Sigma, USA. Cat No.E-8751) in distilled H_2O, for 5 minutes and then destained in distilled H_2O for 5 minutes. DNA fragments were visualised by UV light and photographed using Polaroid 667 film. For visualising small products or resolving small size differences 2% NuSieve GTG low gelling temperature agarose (FMC BioProducts, USA.Cat No. 5008) was used.

Total DNA concentrations were determined by measuring the absorbance at a wavelength of 260nm (A_{260}) using an ultraviolet DU®-64 spectrophotometer (Beckman Instruments, Fullerton, CA). The concentration was then calculated assuming that an A_{260} of 1 is equivalent to a measure of 50 μg/ml of DNA. Specific DNA concentrations were estimated using gel electrophoresis and by comparing the intensity of the ethidium bromide - stained band of interest with that of DNA standards of known concentration.

2.4.3 Size separation of DNA

The sizes of DNA fragments were determined by comparing their relative mobilities in agarose with those of DNA molecules of known sizes (ie DNA molecular weight markers). The molecular weight markers used were : *Hind* III digested bacteriophage lambda (λ) DNA (Bresatec, Adelaide), containing fragments of estimated sizes 23.1, 9.4, 6.6, 4.37, 2.3, 2.0, 0.564, 0.125 Kb; *Eco*RI digested *Bacillus subtillis* phage SPP1 DNA (Bresatec, Adelaide) containing fragments of estimated sizes 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, 0.36 Kb; and *Hpa* II digested plasmid pUC19 DNA (Bresatec, Adelaide) containing fragments of estimated sizes 0.501, 0.489, 0.404, 0.331, 0.242, 0.190, 0.147, 0.111, 0.110, 0.067, 0.034, 0.026 Kb.

DNA samples containing fragments to be separated were electrophoresed until the band of interest was separate from the other DNA. A control track containing a small fraction of the total samples was run adjacent to the sample. This control track was removed from the remainder of the gel, stained with ethidium bromide and the band of correct size was excised from the gel and this lane. The DNA in the sample track was not stained or exposed to UV but was then aligned with the control and the

corresponding area excised and purified according to one of the following methods depending on the size of the fragment to be purified.

2.4.4 Purification of DNA

(A) GENECLEAN®

This method was used to purify specific DNA fragments between 0.5 - 3 kb on the basis of size from other DNA and was carried out as described by the manufacturer (BIO 101, CA, USA). The DNA was separated on an agarose gel and the gel slice containing the DNA fragment was placed into a 1.5 ml eppendorf tube. An equivalent volume of 2.5 - 3 x the weight of the gel slice was added of the supplied sodium iodide (NaI) solution and the agarose melted at 55°C. 5 µl of a Glassmilk suspension was added and placed on ice for 5 - 15 minutes while mixing every 2 - 3 minutes. DNA bound to the silica matrix was pelleted in a microcentrifuge for 5 seconds. The NaI supernatant was removed by washing the pellet 3 times with 700 µl of ice cold "NEW WASH" solution. The pellet was desiccated by heating at 55°C for 2 mins. The DNA was then eluted from the glassmilk with 10 - 20 µl H_2O by heating the tube at 55°C for 10 minutes. The glassmilk was pelleted and the supernatant containing the DNA was removed.

(B) Phenol extraction

This method using phenol/chloroform extraction was used to purify DNA from other reagents during manipulations. The DNA solution was diluted to a total volume of 100 µl with sterile distilled H_2O, and 5µg of glycogen (Boehringer Mannheim, GmbH., Germany) was added. An equal volume of phenol/chloroform (1:1) was added, mixed well and incubated on ice for 3 minutes. The mix was then centrifuged at 1500g for 3 minutes to allow for separation of the different phases.

Most of the aqueous layer containing the DNA was removed. To extract any residual DNA, an equal volume of H_2O this was again added to the remaining phenol mix and incubated on ice for a further 3 minutes prior to microfuging. The aqueous phase was collected as before and pooled together. This was repeated once more. To the total DNA solution collected, 1/10 volume of sodium acetate (NaAc) pH 4.6 and 2 volumes absolute ethanol. The DNA was precipitated by incubation at -20^{0}C for 30 minutes and microfuging for 15 minutes at 4^{o}C. The pellet was washed in 70% ethanol, dried and dissolved in H_2O.

2.4.5 End-filling of DNA fragments

Blunt ended fragments were constructed by end-filling the DNA overhangs after restriction enzyme digestion. The procedure used was similar to that described by Sambrook *et al.,.*, (1989). Klenow polymerase (Pharmacia, Sweden, Cat. No. 27-0928-01) was added to the purified DNA fragment at a concentration of 1 - 2 units/μg of DNA in a 1xTE solution containing $MgCl_2$ and 2'-deoxynucleotide 5' triphosphate (dNTPs) (dATP, dCTP, dGTP, dTTP) (Pharmacia, Sweden. Cat. No. 27-20(5-8)0-02). The final concentration of each dNTP was 1mM and $MgCl_2$ 5 mM in a total reaction volume of 20 μl. The mix was incubated at room temperature for 30 minutes. Klenow polymerase was inactivated by heating at 75^{o}C for 10 minutes. The blunt ended DNA was used directly in ligation reactions (as described in section 2.4.8) but was purified by phenol extraction for use in any subsequent enzymatic reactions.

2.4.6 Dephosphorylation of DNA

To prevent self-ligation, restriction digested DNA fragments and end-filled plasmid DNA were dephosphorylated using calf intestinal alkaline phosphatase (CIP) (Boehringher Manneheim, Germany, Cat. No. 713 023) as described by Sambrook *et*

al., (1989). Digested plasmid DNA was incubated with CIP at a concentration of 1unit/μg of DNA in 1 x CIP dephosphorylation buffer (see appendix 1.2). The reaction was incubated at 37°C for 15 mins or 30 mins for blunt ended or sticky ended DNA fragments respectively. For blunt ended fragments another aliquot of CIP was added at the same concentation as before and the reaction was incubated at 55°C for a further 45 minutes. The CIP was inactivated by heating at 75°C for 10 minutes in the presence of 5 mM EDTA pH 8.0 and the DNA was phenol/chloroform extracted (see section 2.4.3(d)) prior to ligating.

2.4.7 Phosphorylation of DNA

To enable ligation of DNA insert fragments to dephosphorylated vectors, the 5' ends of the insert fragmants were phosphorylated using T4 polynucleotide kinase (PNK) (Pharmacia, Sweden, Cat no 27-0736-01) in a solution containing 66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.1 mM spermidine and 1 mM ATP (Pharmacia, Sweden, Cat. No.27-2056-01), pH 7.4 in a final reaction volume of 10 μl. The reaction mix was incubated at 37°C for 30 minutes. PNK was then inactivated by heating at 65°C for 10 minutes.

2.4.9 Ligation

Ligation reactions were set up with 0.5 - 50 ng of vector DNA and a 1 - 3 fold molar excess of insert DNA in a solution of 25 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.4 mM ATP, pH 7.4 and 2 Units of T4 DNA ligase (Progen, Australia, Cat. No. 700-0000), in a final reaction volume of 10 μl. The ligation reaction was incubated overnight at room temperature (RT). The ligase was inactivated by heating at 65°C for 15 minutes.

2.4.10 Expansion of plasmid DNA

(A) Production of competent bacterial cells

The method used to make E.*coli* DH10β strains (BRL, Cat. No. 8297SA) competent for transformation dercribed below is similar to the protocol described by Sambrook *et al.,* (1989). Bacterial cells from glycerol stocks were streaked on to Ψa agar plates (see appendix 1.3) and left to grow overnight at 37°C. A single colony was used to innoculate 5 ml Luria Broth (LB) medium (see Appendix 1.3) and was incubated at 37°C for 2 hours while being shaken. This was subcultured 1:20 into 100 ml Ψb medium and grown for a further 2 hours. The culture was chilled on ice for 5 minutes and centrifuged in a Beckman J-21C using a JA14 rotor at 1430g for 5 minutes at 4°C. The pelleted cells were resuspended in 40 ml of cold Tfb I Buffer (see Appendix 1.3) and left on ice for 5 minutes prior to pelleting the cells again by centrifugation. The pelleted cells were resuspended in 4 ml of Tfb II Buffer (see Appendix 1.3) and left on ice for 15 minutes. The competent cells were aliquoted into cold 1.5 ml reaction tubes in 200µl lots on ice using a pre-chilled tip. The cells were snap frozen in dry ice and stored at -70°C till required.

(B) Transformation of competent cells with DNA

Competent cells stored at -70°C were allowed to thaw on ice and 100 µl aliquots were placed in cold 1.5 ml eppendorf tubes. To the cells a maximum of 10 ng of DNA (from a ligation reaction or a DNA extraction) was added and the cell/DNA mix was incubated on ice for 30 minutes. The cells were heat-shocked for 90 seconds in a 37°C heating block and immediately placed on ice for 2 minutes. Various dilutions of the reaction mix were spread onto LB plates (see appendix 1.3) containing 100 µg/ml ampicillin (Sigma, USA, Cat. No. A-9518).

(C) Small scale plasmid DNA preparations

This method of plasmid extraction is a modification of the alkali-lysis method described by Sambrook *et al.,* 1989. A single colony was isolated and used to innoculate 2 ml of LB containing 100μg/ml ampicillin overnight at 37°C with shaking. The culture was chilled on ice and 1.5 ml was micrfuged at 3500g in a 1.5 ml reaction tube for 5 mins at 4°C in a microcentrifuge. The supernatant was aspirated and the pellet resuspended in 100μl of solution 1 (50mM glucose, 25mM Tris-HCl pH 8 and 10mM EDTA pH 8). This was mixed by vortexing and left on ice for at least 5 minutes. To each tube on ice, 200μl of freshly made solution 2 (0.2M NaOH and 1% sodium dodecyl sulphate (SDS) made by diluting stock solutions of 2M NaOH and 10% SDS in MQ-water) was added and mixed by inverting the tube. The mixture was left on ice for exactly 4 mins, after which 150μl of solution 3 (made by adding 30ml of 5M potassium acetate and 5.75ml glacial acetic acid to 14.5 ml MQ-water) was added and mixed by inverting the tube. The mixture was left on ice for at least 10 mins. To purify the DNA 500μl of a 1:1 phenol/chloroform solution was added, vortexed and then centrifuged at 1500g for 5 mins at RT. The aqueous phase (~400μl) was transferred to another tube, 240μl of cold isopropanol was added and left on ice for 10 mins. To precipitate the DNA, the mix was centrifuged at 9000g at 4°C for 20 mins. The pellet was washed in 70% ethanol, dried under vacuum and resuspended in 50 μl of TE pH 8.0.

(D) Large scale plasmid preparation

Large scale plasmid preparations were conducted using the materials supplied as part of the QIAquick gel extraction Kit (Qiagen Pty. Ltd., Cliffton Hill, Victoria, Cat. No. 28704) according to manufacturer's protocols for Midi / Maxi plasmid / cosmid purification. A single isolated colony was used to innoculate 10 ml of LB

containing 100 μg/ml ampicillin and the culture was grown to log-phase. This was sub-cultured into 500 ml LB containing 100 μg/ml ampicillin and grown overnight at 37°C with shaking. The culture was placed in two 250 ml centrifuge buckets and centrifuged at 1430g for 20 minutes in a pre-cooled Beckman J-21C centrifuge using a JA14 rotor. The supernatant was discarded and each pellet completely resuspended in 10 ml of Buffer P1. The solutions were transferred to two 35 ml tubes and 10 ml of Buffer P2 was added to each, mixed by inversion and incubated at room temperature for 5 mins. To this, 10 ml of cold Buffer P3 was added, mixed and left on ice for 20 mins. Chromosomal DNA was pelleted by centrifuging at 20,000g in a Beckman J-21C centrifuge using a JA20 rotor for 30 mins at 4°C. The supernatant was collected carefully, transferred to 50 ml polypropylene tubes (Corning, NY, 25331-50) and re-centrifuged for 15 mins at 4°C. The phases were separated by centrifugation at 2000g for 10 minutes in a Beckman J-6B centrifuge. During this time, a QIAGEN-tip 500 column was eqiulabrated by allowing 10 ml of Buffer QBT to flow through it. The supernatant containing the DNA was loaded onto this column. The column was washed twice with 30ml of Buffer QC and the DNA eluted with 15ml of Buffer QF. The DNA was precipitated by the addition of 0.7 x the volume (of the elutant) of isopropanol and centrifugation at 15,000g for 30 mins at 4°C. The pelleted DNA was washed with 5ml of 70% ethanol, air dried and resuspended in 1ml of MQ-water.

This procedure was also used for midi-preps using 100ml cultures and decreased volumes of the same solutions.

2.5 PROCEDURES FOR DNA ANALYSIS

2.5.1 Oligonucleotides

(A) Oligonucloetide Primers

Table 2.1 summarises the location of the oligonucleotide primers used to sequence or amplify human c-*kit*, murine *c-myb*, the gene encoding *neo* resistance or a sequence specific to the Y chromosome.

Table 2.1: Oligonucleotide Primers

name	oligonucleotide sequence	location	orientation
1002 (kit)	5'-GATAGTACTAATGAGTACATGG-3'	2167-2188	sense
SRC-05 (kit)	5'-GAATGGTCTACCACGGGC-3'	2865-2882	anti-sense
SRC-09 (kit)	5'-TTGGCAGCCAGAAATATC-3'	2400-2418	sense
SRC-10 (kit)	5'-AACTTAGAATCGACCGGC-3'	2642-2660	anti-sense
Myb-A (myb)	5'-GGTAATGCCTGCTGTCCCTT-3'	800-820	anti-sense
Myb-S (myb)	5'-ATCTCCAGTCACGTTCCCTAT-3'	965-985	sense
Neo-1 (neo)	5'-CTGAATGAACTGCAGGACGA-3'	1720-1739	sense
Neo-2 (neo)	5'-TGCCTGCTTGCCGAATATCA-3'	2121-2140	anti-sense
Zfy1 (Y)	5'-AAGATAAGCTTACATAATCACATGGA-3'	1491-1516	sense
Zfy2 (Y)	5'-CCTATGAAATCCTTTGCTGCACATGT-3'	2083-2108	anti-sense

The location of these oligonucleotide primers is based on the c-*kit* sequence published by Yarden *et al.*, (1987), the *c-myb* sequence published by Gonda *et al.*, (1985), the *neo* sequence published by Southern and Berg *et al.*, (1982) and the Zfy sequence published by Ashworth *et al.*, (1989) from the sex-determining region of the Y chromosome (Page *et al.*, 1987) .

(B) Purification of oligonucleotide primers

The neo and Y chromosome primers were previously synthesised by Dr. T Gonda. The myb and kit primers were synthesised on a Applied Biosystems 391 DNA synthesiser in the Department of Haematology by Mr. A. Mangos.

Primers were removed from the synthesis columns by elution. To one end of the column an empty 1 ml syringe was attached and to the other end another 1 ml syringe containing 500 µl of ammonium hydroxide (25% ammonia solution, MERCK, Cat. No. 1.05428) was joined. The syringes were used to flush the column with ammonium hydroxide a number of times and left to stand containing the solution for 20 minutes. The solution was then drawn into one syringe and collected. The above procedure was repeated three more times until a volume of 2 ml was obtained. The primer/ammonium hydroxide solution was incubated overnight at 56°C. The vials were allowed to cool and 18ml Butanol was added to the primer mix. The primer was precipitated by centrifugation at 700 g for 20 mins, the pellet washed with 70% ethanol, dried and resuspended in 200µl of MQ water. The concentration was calculated from the Absorbance determined by spectrophotometry. The molarity was calculated using the formula: concentration of oligonucleotide(M) = (x µg x 10^{-6}g ÷ total mwt for oligonucleotide) ÷ 1 x 10^{-3}. The molecular weight (mwt) of the oligonucleotide calculated as the sum of the dNTPs (where the mwt of each A=347.2, T=332.2, G=363.2, C=323.2).

2.5.2 Polymerase Chain Reaction (PCR)

All reagents were aliquoted using non-aerosol tips in order to prevent contamination and either 25µl or 50µl reactions were set up in 0.5 ml

micro-centrifuge tubes (Edwards Medical Supplies, NSW, Australia. Cat. No. 3020-500).

Each reaction mix contained 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, dATP, dGTP, dCTP and dTTP at 200 mM each (Cat. No. 27-20(5-8)0-02), 1 μM of each oligonucleotide primer, 2.5 U AmpliTaq DNA polymerase (Perkin Elmer Cetus, USA, Cat. No. N801-0060), 0.5 ng template DNA and sterile Milli-Q H_2O to make up the final volume. Each reaction mix was overlayed with 50 μl of mineral oil.

DNA was amplified using a Perkin Elmer Cetus thermal cycler by initially denaturing the DNA at 94°C for 7 minutes; followed by 25-35 cycles each consisting of a denaturation, annealing and extension (see below); followed by a final extension at 72°C for 7 minutes. The temperature used for the annealing of primers was between 2-5°C lower than the average melting temperature (Tm) of the two oligonucleotides. The Tm of each oligonucleotide was calculated using the formula: Tm = 4x (G+C) + 2x (A+T)). The temperature was optimised for each set of primers and the conditions that were used for the PCR reactions are listed below.

Primers	Denaturation	Annealing	Extension	Number of cycles
1002 and SRC-05	1min / 94°C	1min / 56°C	1min / 72°C	30
SRC-09 and SRC-10	1min / 94°C	1min / 52°C	1min / 72°C	25
Myb-A and Myb-S	1min / 94°C	1min / 61°C	1min / 72°C	35
Neo-1 and Neo-2	1min / 94°C	1min / 55°C	1min / 72°C	35
Zfy-1 and Zfy-2	1min / 94°C	1min / 60°C	1min / 72°C	30

2.5.3 Sequencing of DNA

The Dideoxy method used for sequencing DNA was performed using the Super-Base Sequencing Reagent Kit (Bresatec, Adelaide, Aust. Cat. No. SBK-2) essentially according to manufacturer's protocol.

(A) Preparation of DNA

DNA used for sequencing was prepared differently to the methods outlined above. A single transformed bacterial colony was grown in 5ml of 2YT medium (see appendix 1.3) overnight at 37°C with shaking. The culture was chilled on ice for 5 mins and 1.5ml was transferred to a sterile eppendorf tube. The cells were pelleted by microfugation at 9000g for 1min. The cell pellet was resuspended in 250µl of lysing buffer (50mM Tris-HCL pH 7.5, 62.5mM EDTA pH 8, 0.4%Triton X100 and 2.5M LiCl in H_2O) and left on ice for 5 mins after vortexing. To this, 20µl of 10mg/ml lysozyme was added and left on ice for a further 2 mins. The lysate was boiled for 1 min in a waterbath, placed on ice for at least 15 mins and centrifuged at 9000g for 30 mins at 4°C. The supernatant was collected, 500µl of cold 100% ethanol was added and centrifuged at 9000g for 15 mins at 40C. The DNA pellet was washed with 1 ml of cold 70% ethanol, air-dried and resuspended in 30µl of sterile MQ.

(B) Incorporation of labelled nucleotides to sequence DNA

Sequencing of double-stranded DNA was performed using the Super-Base Sequencing Reagent Kit (Bresatec, Adelaide. Cat. No. SBK-2) according to manufacturer's instructions.

The template DNA was first denatured by alkaline treatment, by adding 4µl of 2M NaOH to 10µl of the plasmid prep in a final volume of 20µl made up with sterile MQ water. This was incubated at room temperature for 10 minutes. The DNA was then precipitated by adding 6µl of 3 M NaAc pH 4.5, 120 µl of Ethanol and 14µl of sterile MQ water, incubating at 70°C for at least 15mins and centrifuging at 13,000rpm for 10 mins at 4°C. The DNA pellet was washed with 70% ethanol and vacuum-dried.

The DNA was resuspended in a primer annealling mix containing 2µl 5x annealing buffer, 100ng primer in a final volume of 10ml made up with MQ water. This was mixed well and incubated at 37°C for 30 minutes and then at RT for 20 minutes. To the annealed primer/template mix were added 2 µl diluted labelling/extension mix, 1 µl 100mM 1,4-Dithiothreitol (DTT), 1µl α-^{35} S-dATP (10 µCi) (Bresatec, Adelaide, Cat No. SDA-2), 1µl MQ-water and 1µl T7 DNA Polymerase (diluted to 2 Units/µl) (Pharmacia, Sweden, Cat. No. 27-0985-04). To the wells of a micro tray (Disposable Products, Australia, Cat. No. 239728) were added 2.5µl of the provided A,G,C and T termination solutions for each clone to be sequenced and these were prewarmed to 37°C on a heat block. To the DNA/primer mix was added the enzyme and the reaction incubated for 4-5 minutes prior to transferring 3.5µl of each labelling/extension reaction mix to each of the corresponding wells of the microtitre tray. These were incubated for a further 4-5 minutes prior to the addition of 4 µl of stop/loading buffer. The reactions were either analysed on the same day or stored at -20°C for up to a week. The samples were denatured at 95°C for a few minutes on a heat block befor being loaded onto the gel.

(C) Sequencing Gel Electrophoresis

A sequencing gel mix containing 35 ml of 46% Urea (see appendix 1.4), 28 ml of 20% acrylamide (see appendix 1.4), 7 ml of 10 x TBE (see appendix 1.4), 350 µl of 10% ammonium persulphate (Sigma, USA, A-9164) and 70 µl of TEMED (Sigma, USA, T-8133) was made fresh, and immediately poured in between two taped and clamped glass plates (42.5 x 33.5 cm and 39.5 x 33.5 cm - the smaller glass plate was coated with Coatasil (Ajax Chemicals, Cat. No. 2293)) using a 19 gauge needle. The solution was allowed to set for at least 20minutes before gel was pre-electrophoresed

for 30 minutes at 1800V. The wells were flushed using TBE buffer and 2µl of each denatured sample was loaded. The gel was allowed to electrophorese in 1xTBE buffer at 1700V for various periods of time depending on the position of the sequence to be read. If a longer DNA sequence was required then samples were loaded at staggered time intervals.

Following electrophoresis the plates were separated and the gel was fixed for 30 minutes in a solution of 10% methanol and 10% acetic acid in MQ-water. The gel was then placed over a Whatman 3M filter paper of the same size and dried under vacuum at 80°C for 40 minutes. The dried gel was autoradiographed overnight at -70°C.

2.5.4 Southern Blotting

(A) Extraction of genomic DNA

Cells were collected, washed once with PBS and used directly for extrating DNA or were snap frozen in liquid nitrogen and stored at 70°C till needed. The cell pellet was resuspended in 1ml of buffer containing 0.1M NaCl, 50mM Tris pH 7.5 and 1mM EDTA pH 7.5. In quick succession, proteinase K was added to 500mg/ml followed by SDS to 0.5%(w/v) and RNase to 100 µg/ml by the addition of stock solutions. The suspension was incubated at 37°C for 1hr and SDS was added to 1%(w/v). Genomic DNA was purified from the suspension by phenol / chloroform extraction followed by chloroform extraction (Sambrook *et al.,* 1989 and as described above). The DNA was isolated by overlaying with twice the volume of 100% (v/v) ethanol and spooling the DNA from the interface using a glass rod on which the DNA was washed twice with 70% (v/v) ethanol and dissolved in 1xTE by mixing overnight.

(B) Preparation of DNA, Gel electrophoresis and Transfer of DNA

20μg of total genomic DNA from each sample was digested with the restriction endonuclease Xba 1 (Pharmacia Biotech) for 16 hours at 37°C in a 3 fold excess of enzyme according to manufacturer's instructions. A small aliquot of each sample was tested for complete digestion, and further enzyme was added to partially digested samples and incubated for 2-5 hours. 10μg of each digest sample was purified by ethanol precipitation (as outlined previously), resuspended in 1xTE and separated on a 0.8% agarose gel by electrophoresis at 30 volts for 16 hours. The DNA was denatured by washing the gel for 45 minutes in 1.5M NaCl and 0.5M NaOH, after which it was neutralized in a solution of 1M Tris pH 7.4 and 1.5M NaCl for at least 30 mins. DNA was then transferred to a Hybond^{TM}-N nylon membrane (Amersham, RPN 303B) filter by capillary action overnight (as outlined Sambrook *et al.*, 1989) using 10xSSC (see appendix 1.5). The filters were irradiated with 0.75J/cm^2 ultraviolet light to crosslink the DNA. Filters were stored at room temperature until required for probing.

(C) Southern Blot Hybridization

Prior to probing, filters were wet in 2xSSC and between plastic membranes (Hybaid Ltd., Middlesex, Eng), then placed in a roller bottle (Hybaid Ltd., Middlesex, Eng) and heated to 42°C in a Hybaid oven (Hybaid Ltd., Middlesex, Eng) while rotating. The filters were prehybridized in 20ml of pre-hybridization solution (see appendix 1.5) for at least 3 hrs at 42°C while rotating in the Hybaid oven.

During this time a radiolabelled probe was prepared. Radiolabelling of DNA probes was performed by the random priming technique using the Megaprime DNA labelling kit (Bresatec, Adelaide, Aust.) following the recommended protocol. To

50ng of DNA, 6µl of Decanucleotide mix was added and diluted in MQ-water to 12µl. The DNA was denatured at 100°C for 10mins and cooled. In the following sequence 6µl nucleotide buffer 2A, 5µl of 50mCi ^{32}P-dATP (Bresatec, Adelaide. Cat. No. ADA-3) and 1µl of (5U) Klenow enzyme were added. The reaction was incubated at 37°C for 30mins and terminated by the addition of 5µl of 10mg/ml Proteinase K and 10µl of 0.1M EDTA. The probe was purified using a MicroSpin™ S-300 HR column (Pharmacia Biotech, USA. Cat. No. 27-5130-1). The column was prepared by vortexing and centrifugation at 735g for 1 minute to set up the sepharose column. The probe was then placed onto the column and collected in an eppendorf tube by centrifugation at 735g for 5 mins. The probe was denatured at 100°C for 10mins. The specific activity (SA) of the probe was calculated by measuring the total counts of a 5µl diluted sample of probe using a Bioscan QC2000 beta counter and using the following formula : SA = counts/min x dilution factor x total volume of the probe mix.

The filters were placed in another aliquot of the pre-hybridization solution and the radiolabelled probe was added such that the count/min for the total volume was between $2x10^7$ and 10^8 . The probe was incubated with the filters at 42°C overnight while rotating. The following day the probe was discarded and the filters rinsed in 2xSSC. The filters were removed from the roller bottles and washed to remove non-specific bound probe. The filters were washed once at room temperature for 30 mins in 2xSSC and 0.1%SDS and twice at 50°C for 30 mins in 0.1xSSC and 0.1%SDS. The filter was then drained and autoradiographed at -70°C for 1day-1week. In some cases, as indicated, a Phosphor-imager (Molecular Dynamics, Sunnyvale, CA) was used to detect and quantitate the hybridized radioactive emission of specific DNA fragments.

2.6 RNA ANALYSIS

For all RNA procedures materials were kept 'RNase-free'. All MQ-water used was treated with 0.1%v/v diethyl pyrocarbonate (DEPC) (Sigma, USA, Cat.No. D-5758) at 37°C overnight and autoclaved to inactivate the DEPC. All apparati were handled with gloves and glassware was heated in a 180°C oven overnight prior to autoclaving.

2.6.1 Extraction of total RNA

Cells were washed twice in PBS and either used directly for extraction or cell pellets were snap frozen in liquid nitrogen and stored at -70°C till needed. Total RNA was prepared using the RNAzol™ B RNA isolation solvent (Tel-Test Inc., Texas, USA, Cat. No. CS-104) according to manufacturer's instructions. Fresh or frozen cell pellets (containing $5x10^6$ cells) were resuspended in 1ml RNAzol solution and mixed by pipetting. 100µl of chloroform was added and left on ice for 5 mins. The mixture was centrifuged at 9000g for 15mins at 4°C in a microfuge to separate the phases. The aqueous layer was collected and an equal volume of isopropanol was added at left at 4°C for 45mins. The RNA was precipitated by centrifugation at 9000g for 15mins at 4°C. The pelleted RNA was washed with 1ml of 70% ethanol, dried under vacuum and resuspended in MQ-water. The concentration of RNA was calculated from the A_{260} determined using a spectrophotometer (A_{260} of 1 was assumed to be equivalent to a concentration of 40µg/ml).

2.6.2 cDNA synthesis

Total RNA was used as template to synthesis cDNA using the cDNA first strand synthesis kit (Pharmacia Biotech, USA, Cat. No. 27-9261-01) according to manufacturer's instructions. 4µg of total RNA was diluted in MQ-water to a volume of 8µl and incubated at 65°C for 10 mins. To each reaction was added 5µl of 1st strand

mix, 1µl of DTT and 1µl of random primer. The cDNA was synthesized at 37°C for 1 hour. The RNA/cDNA mix was denatured at 90°C for 5 mins before use in a PCR reaction. The reactions were set up as described in section 2.5.2 using 5µl of the cDNA mix as the source of template DNA in a 50µl PCR reaction.

2.7 ANALYTICAL PROCEDURES FOR PROTEINS

2.7.1 Western Blotting

(A) Whole cell protein extracts

Cells were washed twice in cold PBS, pelleted and resuspended in lysis buffer to a final concentration of 10^7 cells/ml. To 10ml of freshly made Lysis buffer (see appendix 1.6), 200µl of Phenylmethyl-sulfonylfluoride (PMSF) (see appendix 1.6) and 100µl of a mix of other protease inhibitors (see appendix 1.6) were added immediately prior to use. Cells were sonicated for 5 seconds at the output control 3 using an Ultrasonic cell disrupter (Microson™, USA) while being kept on ice. The lysate was boiled for 3 minutes and centrifuged at 9000g for 45 minutes at 4°C to precipitate genomic DNA and cellular debris. The supernatant was aliquoted and stored at 20°C.

(B) Gel electrophoresis

An 8% SDS polyacrylamide mini-gel was set up using the hsi (mighty small II) multiple gel electrophoresis apparatus (Hoefer Scientific Instruments, CA, USA, Model SE 200/ SE 250/ SE 260). The gel mix was prepared from stock solutions (see appendix 1.6) and immediately poured between two plates (8 x 10cm) kept apart by 1mm spacers placed in a multiple gel caster. When the gel was polymerised the combs were placed over the top and freshly made 5% polyacrylamide stacking gel mix was poured over the gel to create loading wells. When the stacking gel was polymerised

the plates were placed in a multiple gel electrophoresis unit and filled with running buffer (see appendix 1.6). A 10µl aliquot of each sample to be analysed was diluted 1:2 in 2x loading buffer (see appendix 1.6), boiled for 5mins, loaded onto the mini-gel and electrophoresed at a constant current of 20mA.

The proteins from the gel were then transferred to a filter using a Tranphor electrophoresis system (Hoefer Scientific Instruments, CA, USA, Model TE 22) according to instructions. A Polyvinylidenedifluoride (PVDF) membrane (Micron Separations Inc., Westborough, MA) was cut to size of (8 x 5cm), placed in methanol and soaked in transfer buffer (see appendix 1.6) for at least 15mins. Two pieces of Whatman 5mm paper were also cut to the same size and wet in transfer buffer. The gel (without the stacker) was placed over the filter and the Whatman paper either side of the two. The layers were placed between 2 sponges and put into a plastic case that was placed in the transfer apparatus containing transfer buffer. The proteins were transferred overnight with a current of 30mA. The filter was removed and probed directly or air-dried and stored at room temperature.

(C) Detection of proteins by probing with antibodies

The membrane was blocked for 2 hours at room temperature in 5% (w/v) skim milk powder in 1xTBS (see appendix 1.6) while rocking. The filter was probed with the primary antbody diluted in TBS-Tween (0.1% Tween in 1xTBS) for 2 hours at room temperature. The filter was then washed 1x 15 minutes, and 2x 5 minutes in TBS-Tween at room temperature. For each susequent step the filters were probed for 1 hour at room temperature and washed as above. Horseradish peroxidase (HRP) conjugated reagents were detected using an Enhanced Chemiluminesence (ECL) kit (Amersham Life Sciences, Burkinghamshire, Eng., Cat. No. RPN 2106) according to the manufacturer's instructions, and the filter was autoradiographed at room

temperature for a maximum of 30 minutes. Alkaline Phosphatase (AP) conjugated reagents were detected using a Western Blotting Kit (Vistra Systems, Amersham, Burkinghamshire, Eng., Cat. No. RPN 5780) and the filter was scanned using a Fluoro-Imager (Molecular Dynamics, Sunnyvale, CA)

To detect Myb proteins, mAb 5.1 directed against the amino terminal region was used as the primary antibody. Ascites containing 5.1 mAb was diluted 1:500 and biotin-conjugated 5.1 was diluted 1:1000. The 5.1 antibody was detected by probing with anti-mouse Ab - HRP (diluted 1:5000) and the biotinylated 5.1 was detected with strepavidin-AP (diluted 1:500).

2.7.2 Other methods

Proteins were also detected by immunofluorescence (as described above in section 2.3.2) or immunocytochemistry (as described above in section 2.3.3A).

2.8 INTRODUCTION OF DNA INTO EUKARYOTIC CELLS

2.8.1 Recombinant DNA cloning into retroviral expression vectors

Specific purified cDNA fragments were cloned into the polylinker of the retroviral expression vectors RED (Hu *et al.,* 1991) or RUF (Rayner and Gonda, 1994). as described in more detail in the following chapters. The retroviral expression plasmid vectors pRUF(NL) and pRUFneo are shown in Figure 2.1. DNA plasmids containing retroviral expression contructs were expanded by large scale preparations and transfected directly into Ψ2 packaging cells (Mann *et al.,* 1983). The Ψ2 cells produce empty ecotropic retrovirus. Upon plasmid transfection, the introduced retroviral constructs are packaged into these viral particles. Transfectants are used as a source of retrovirus carrying the construct to infect other target cells.

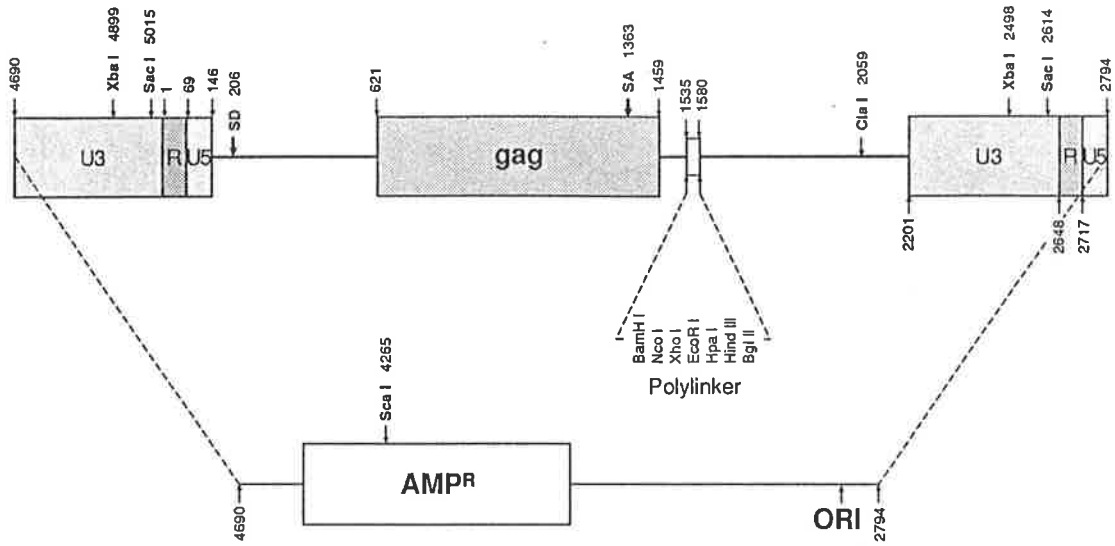
Figure 2.1: The pRUF(NL) and pRUFNeo retroviral expression vectors.

The nucletides in the plasmid sequence are numbered at various points. The splice donor and splice acceptor sites (SD and SA respectively) and restriction endonuclease cleavage sites are labelled.

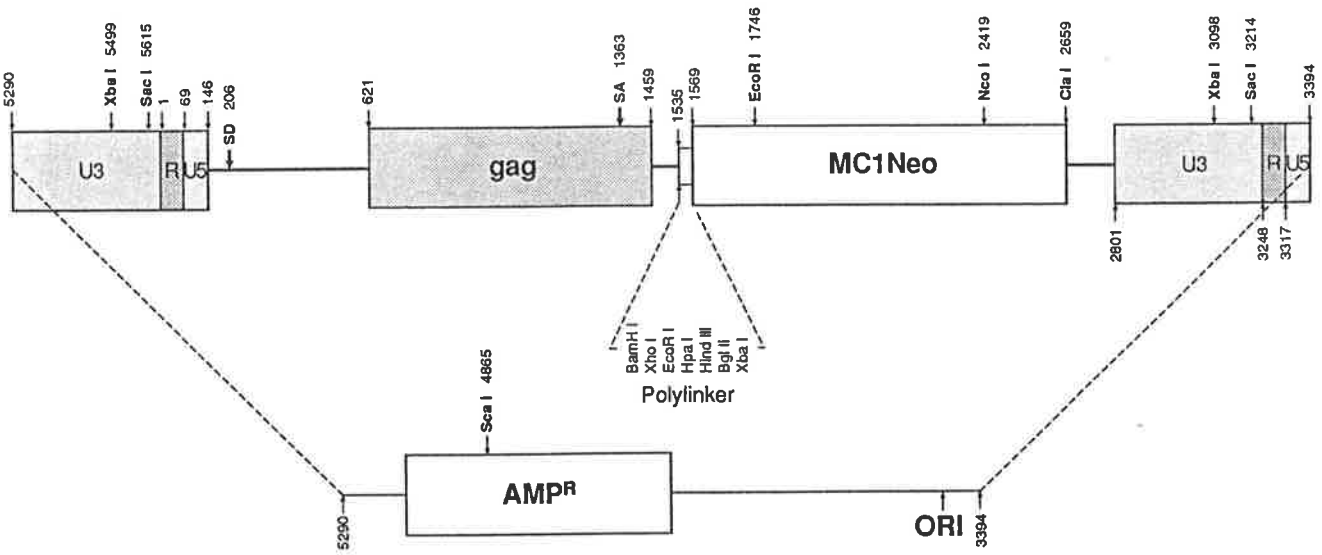
All cleavage sites in the polylinker of pRUF(NL) are unique. In the polylinker of pRUFNeo, BamHI, XhoI, HpaI, HindIII and BglII are unique. In the LTRs KpnI and SmaI sites are not shown. A unit proviral length is 2648bp in pRUF(NL) and 3259bp in PRUFNeo. Sequences in pRUF were derived mostly from MoMLV, with some sequences from MPSV (nucleotides 5015-1535 in both vectors, 2059-2614 in pRUF(NL) and 2670-3225 in pRUFNeo. The pRUF(NL) vector was derived from pRUF by the insertion of a sequence (including an NcoI site) between the BamHI and XhoI sites. The pRUFNeo vector was derived from pRUF by the insertion of an MC1neo cassette between the BglII and ClaI sites of pRUF.

U3, R and U5 represent repeat sequences within the LTR. AMP^R represents the cDNA encoding resistance to Ampicillin. Ori represents the origin site of plasmid replication.

pRUF(NL) (5050 bp)



pRUFNeo (5650 bp)



2.8.2 Calcium phosphate transfection into Ψ2 packaging cells

The viral packaging fibroblast cell line Ψ2 was transfected with retroviral plasmid constructs as described by Lang *et al.,* (1985). Briefly, a day prior to transfection, 6cm dishes were each seeded with 2 x 10^5 Ψ2 cells. On the day of transfection a co-precipitate was formed by mixing two solutions. In a 10ml polystyrene tube 62.5µl 2M $CaCl_2$ and 20 µg plasmid DNA made up to a final volume of 500 µl with sterile tissue culture grade Milli-Q-purified H_2O was mixed. To a 20ml conical polystyrene tube, 62.5 µl 2M NaCl, 7.5µl 0.1M NaH_2PO_4, pH 7.0, 50µl 0.5M HEPES, pH 7.1 was mixed and made up to a final volume of 500µl with H_2O. This solution was added dropwise to the DNA mix whilst bubbling air through a glass pipette into the same tube. The mix was left to stand at RT for 30 minutes until a fine precipitate was formed. A volume of 500 µl of this co-precipitate was added to each dish of duplicate dishes and incubated at 37°C in 5% CO_2. After 24 hours, the medium was aspirated from the dishes and the cells were 'glycerol shocked' with 1 ml of DMEM containing 15% glycerol for precisely 4 minutes while rocking gently every 30 seconds. This was removed and the cells were washed with 5ml of DMEM and then 4ml of fresh DMEM/10% FCS was added. The following day the cells were harvested and subcultured either 1:10 - 1:100 in DMEM /10% FCS containing 400µg/ml G418. The medium was substituted with fresh stocks twice a week and when cells transfected without DNA had died (10 - 12 days after G418 selection), populations were harvested as mixed 'pools' or individual clones were isolated. These selected Ψ2 cells were used as a source of retrovirus.

2.8.3 Retroviral infection of cells

(A) Infection of adherent cells using supernatant

Virus containing supernatant was collected from Ψ2 transfected cell lines by adding fresh medium (without any G418) to almost confluent monolayers. The following day the medium was collected and filtered through a 0.45 μm filter (Sartorius, Germany, Cat. No. 165 55K). The supernatant was used fresh or frozen at -70^{0}C in 1ml aliquots and thawed immediately before use. The cells to be infected were seeded into tissue culture dishes (60 mm in diameter) 24 hours prior to infection such that the cells were at 10 - 20% confluent on day of infection. The medium from the dishes was removed and 1 ml of neat or diluted (1:10 and 1:100) viral supernatant containing 4μg/ml polybrene was added. The dishes were incubated at 37^{o}C, rocking every 30 minutes and after 2 hours, 3ml of fresh DMEM / 10% FCS was added. After 48 hours the cells were harvested and subcultured (1:10, 1:20 and 1:100) into 60 mm diameter tissue culture dishes in DMEM/10% FCS containing 400μg/ml of G418. The culture medium was replaced twice weekly and G418 selection was maintained until un-infected cells were dead.

To determine the titre of the virus producing lines, NIH-3T3 cells were infected with virus-containing supernatant and the dishes were stained with Giemsa (as follows) when separate colonies were visible. The medium was removed and the cells fixed to the dish with 4% formaldehyde for 10 minutes. The fixative was removed and the dishes were incubated with a 10% Giemsa solution for 20 minutes then rinsed in distilled water. After air-drying the dishes, the number of colonies was counted. The viral titre was calculated using the formula: Titre (colony forming units (CFU)/ml) = number of G418^r colonies x dilution of viral supernatant x dilution of cells.

(B) Infection of suspension cells by co-cultivation

Semi-confluent monolayers of G418 selected Ψ2 transfectants were irradiated at 30 Grays, harvested and seeded at 1 x 10^6 in a 25 cm^2 flask. Non-adherent cells to be infected were washed and added at various cell densitites depending on the cell line used or the availability of primary cells. DMEM containing 10% FCS and growth factors (as indicated) was added to each flask to a final volume of 5ml and the cells were co-cultivated for 2 days. The non-adherent cells were collected, washed and cultured in 5ml of DMEM /10% FCS /growth factors. Infected cells were selected in medium containing up to 1mg/ml of G418, which was replaced every 3-4 days until un-infected cells had died.

To assess the titre of transfected Ψ2 cells producing virus that did not contain a neo^R marker, $2x10^5$ FDC-P1 cells were infected by co-cultivation with $5x10^5$ Ψ2 transfectants in a 15 cm^2 tissue culture dish containing 4ml of medium as described above. Following infection the FDC-P1 cells were harvested and expanded for 3 days in 15ml of medium. The cells were collected and genomic DNA was extracted, prepared and used for Southern Blotting as described in section 2.5.4. A Phosphor-Imager was used to quantitate the intensity of *myb* bands and the relative titre expressed as a ratio of intensity of the retroviral *myb* band to the endogenous *myb* band.

2.9 ANALYSIS OF CELLULAR FUNCTIONS

2.9.1 Cell Growth and Proliferation Assays

(A) Analysis of cell number by Absorbance.

This procedure was carried out using the CellTitre™ Assay reagents (Promega, WI,

USA) according to manufacturer's instructions. The procedure is based on the cellular conversion of a tetrzolium salt to a coloured formazan product that is measured by Absobance at 570nm and used as an assessment of cell number.

The cells to be assayed were harvested, washed twice in HBBS and resuspended in DMEM /10%FCS to a concentration of $2x10^4$ - $4x10^5$ cells/ml. Duplicate 50µl aliquots of the cell suspension were placed into the wells of a 96 well tissue culture tray containing 50µl of medium plus the relevant growth factors and incubated for 2 - 6 days. At each time point to be monitored, the tray was assayed for total cell density. To each well, 15µl of dye solution was added and incubated at 37^0C. After 4 hours, 100ml of the Solubilization/Stop solution was added to each well and incubated at 37^0C for 1 hour. The Absorbance at 570nm was measured using an ELISA plate reader (Biorad).

(B) Analysis of Cell Survival, Growth and Proliferation by Flow Cytometry

This assay and the analysis of the data were carried out essentially as described previously by Ashley *et al.,* (1994). The procedure is based on the incoporation of a fluorescent lipophilic dye into the cell membrane. The level of fluorescence is measued at the start of the assay and at other subsequent time-points. During cell division the amount of dye per cell is halved and the level of fluorescence is used to measure the number of divisions undergone by the cells in the population. At each time-point, addition of a standard concentration of beads to the cell population is used to calculate the cell number, which together with the average number of divisions is used to determine the viability of the population.

The cells were labelled as folllows using the solutions provided in the PKH26 Red fluorescent Cell linker kit (Sigma, St. Louis, MO, USA) according to manufacturer's instructions. Cells were washed twice in HBBS, resuspended in

Diluent C to a final concentration of 2 x 10^7/ml and an equal volume of $5x10^{-6}$M lipophilic dye PKH26 was added. After incubation for 2.5 minutes with gentle mixing, an equal volume of FCS was added with incubation for 1minute, followed by the addition of an equal volume of DMEM/10%FCS and incubation for 1 minute. The labelled cells were washed 3 x in DMEM/10%FCS and seeded at $2x10^4$ viable cells/well in duplicate into three 24 well trays. To each well, medium plus growth factors was added to a final volume of 1ml. On the same day (day 0) and at day 2 and day 4, the total cells in each well were harvested, centrifuged and resuspended in 300µl PBS. To each tube, 50µl of Standard-Brite™ calibration beads (Coulter, Hialeah, FL, Cat. No. PN 660414) at a fixed concentration was added. The cell count, bead count and the Mean Fluorescence Intensity (MFI) for each sample was analysed on a Profile II Flow cytometer (Coulter, Hialeah, Florida). The total cell number was calculated using the formula : Cell Density (cells/ml) = (Viable cell count ÷ bead count) × (Volume of the beads ÷ Volume of the cells) × Concentration of the beads. Since each division results in halving of the fluorescence of individual cells, the average number of divisions was calculated based on the MFI at each time point relative to the MFI at day 0. The formula used was: Number of Divisions = (log (MFI at day 0 ÷ MFI at that day)) ÷ log (2). The survival or maintenance as the percentage retention of fluorescence relative to day0 was calculated by the formula: Cell Maintenance = (MFI at that day × total viable cells at that day) ÷ (MFI at day 0 × Total cells at day 0) × 100.

2.9.2 Assessment of Clonogenicity of cells

All cells were plated in a methylcellulose semi-solid medium. Frozen aliquots of prepared methylcellulose (see section 2.1.1) were thawed at 37°C. Depending on the quantity required, for each 10 ml of medium : 8.5 ml of methylcellulose mix, 1.5 ml

of single strength IMDM (prepared by diluting double strength 1:2 in MQ water) and L-Glutamine (2mM final) was mixed together. Cells to be plated in semi-solid medium were collected and washed 3 times in HBBS /2% FCS and resuspended in DMEM /20% FCS. The cells were counted, mixed at a specific concentration with methylcellulose semi-solid medium and growth factors and 1ml aliquots placed in 35mm tissue culture dishes (Corning, NY, USA). The plates were placed in humidified containers and incubated as for cell cultures.

2.9.3 Assessment of Phagocytic capacity

Cells were cultured in tissue culture chambers on chamber slides (Nunc, IL) for 4 days. Non-adherent cells were discarded and adherent cells washed in DMEM. 200ml of DMEM supplemented with 20% non heat inactivated FCS and 2ml of fluorescent latex beads (Fluoresbrite plain YG20 micro spheres, 2.5% solids-latex, diameter 1.8 mm, Polyscience Inc., Warrington, PA) was added to each well and incubated for 2hrs. The chambers were removed and the slides washed thoroughly in PBS prior to mounting. Cells were observed using a confocal microscope (Biorad, Microscopy division, Hertfordshire, England).

2.10 EXPERIMENTS USING MURINE FOETAL LIVER CELLS

2.10.1 Transformation assay

(A) Extraction of FLC

Foetal livers were obtained from foetuses of a day 14 pregnant female CBA mouse using a dissecting microscope. The cells from whole livers were dispersed by pipetting and the cell suspension was washed twice in DMEM /10% FCS. The FLC were either used fresh or cryopreserved.

(B) Infection of FLC

Fresh cells were used directly in a co-cultivation with transfected Ψ2 cells (see section 2.8.3 (B). Frozen cells were thawed on the day of the infection and incubated for 3 hours in DMEM /20%FCS. The cells were then counted and if viability was >50% were used similarly for infection, by co-cultivation with Ψ2 cells. Depending on the availability of FLC, 2 x 10^5 - 1 x 10^6 cells were co-cultivated for 2 days with 1 x 10^6 irradiated Ψ2 cells per flask in DMEM/ 10%FCS containing 500U/ml GM-CSF and 400U/ml IL-3. Duplicate infections were set up for each construct to be assessed. After 2 days the non-adherent cells from each flask, were collected and washed in HBBS.

(C) Assessment of clonogenic potential

The cells collected from each flask were resuspended in 5ml of DMEM/20%FCS/400U/ml GM-CSF and cultured for 1 week. During this time, 1-2ml of extra medium was added to each flask if the cell density was high in any single flask. Following a week in culture, cells from each flask were collected, washed in HBBS and counted. Triplicate aliquots of 2 x10^4 cells were plated in methylcellulose containing 500U/ml GM-CSF. The dishes were incubated for 7 days and the number of colonies formed were counted using an inverted microscope. All colonies containing more than 50 cells were scored.

(D) Maintenance of long term cell lines

At the time of plating, an aliquots of cells from each flask were expanded in liquid culture to give rise to cell lines. The cells that proliferated were maintained as long term lines in DMEM/15% FCS supplemented with 400U/ml GM-CSF where indicated.

2.11 EXPERIMENTS USING MURINE BONE MARROW CELLS

2.11.1 Transplantation Assay for reconstitution

(A) Mice species

For all experiments 8 week old C57BL/6J mice of different congenic strains (Morse HC 1992) were used and housed in the Animal House Quarantine Section (Medical School, University of Adelaide). Mice of the C57BL/6J strain with the *Ly5*^{a} allele of specificity Ly5.1 (also known as the *Ptprc*^{b} allele of specificity CD45.2), obtained from the Animal Resources Centre, Perth, were used as donors. Mice of the C57BL/6J strain with the *Ly5*^{b} allele of specificity Ly5.2 (also known as *Ptprc*^{a} allele of specificity CD45.1), obtained from the Animal Facility, WAITE Institute, Adelaide, were used as recipients.

(B) Extraction of BM cells from donor mice

Donor mice were with treated with Fluorouracil (5FU) (DBL, F.H. Faulding and Co., trading as David Bull Laboratories, Vic., Aust. - product sold for patient treatment). The stock solution of 50 mg/ml was diluted 1:2 in PBS and a volume providing a final dose of 150 mg/kg of the mouse body weight was injected intraperitonealy 4 days prior to the extraction of BM. To obtain BM cells, the donor mice were killed and the femurs removed and placed in a petri dish containing IMDM. The ends of each femur were cut and the BM extracted immediately by flushing through 1ml of IMDM using a 21g needle and 1ml syringe. The BM cells were dispersed in the medium by pipetting and the tissue debris was allowed to settle. The cell suspension was decanted and diluted in IMDM /20% FCS.

(C) In vitro manipulation of BM cells

The BM cells were counted and the total cells extracted from one femur was co-cultivated with 1 x 10^6 transfected Ψ2 cells in a 25 cm^2 flask containing 5ml of

IMDM /20%FCS / 500U/ml IL-3 / 100ng/ml muSCF / 10ng/ml IL-6. Various experiments were assessed for the optimal survival of recipients, the highest proportion of donor cells and the greatest efficiency of repopulation with infected cells. Initial expreriments tested an infection protocol in which BM cells were 'pre-cultured' in the medium containing cytokines (as above) prior to co-culture, (since this was believed by other researchers to enhance the rate of retroviral infection). The experiments tested included : pre-culture in cytokines for 1 day and co-culture for 2 days, pre-culture for 1 day and co-culture for 3 days, pre-culture for 2 days and co-culture for 2 days or co-culture for 3 days. Following infection, the non-adherent cells were harvested, washed in IMDM /2% FCS and resuspended in IMDM. A fraction of each culture (1/10) was used to assess transformation in an *in vitro* assay and the rest were used to reconstitute the BM of recipient mice.

(D) Irradiation and of recipient mice and transplantation of donor cells

On the day of transplantation, recipient mice were lethally irradiated. The mice were exposed to split dose of 5 Grays each, 4 hours apart, from a Cs-137 source (in an IBL Blood Product Irradiator - in the Blood Transfusion Unit of the Royal Adelaide Hospital). The irradiated recipients were injected intravenously with infected BM cells in order to reconstitute their haemopoietic systems. Each recipient was intravenously injected with the cells derived from one donor femur. All intravenous injections were performed by Velta Vingelis (Dept. of Genetics, University of Adelaide).

2.11.2 Analysis of blood from transplanted mice

At various times post-transplantation, peripheral blood samples were obtained from the retro-orbital plexus of recipient mice. Samples of 150-200µl were placed immediately into 4ml EDTA pre-treated tubes (Griener labortechnik, Vacuette K3E)

to prevent clotting. All blood samples were obtained with help from Velta Vingelis (Dept. of Genetics, University of Adelaide).

(A) Analysis of white cell count and proportion of donor cells

In order to analyse cells by flow cytometry, red blood cells were lysed by the addition of 1ml fresh lysis buffer (see Appendix 1.6) to 100µl of whole blood for 10 mins on ice. White blood cells were centrifuged and resuspended in 100µl PBS.

To determine the cell count, 50µl of the Standard-Brite™ beads (see section 2.9.1B) were added to 25µl of the white cells. The cells/beads were analysed on the Profile and the cell number calculated as outlined in section 2.9.1B.

To determine the proportion of donor derived cells in the peripheral blood of the recipients, 25µl of white cells were incubated with 2µl of FITC conjugated antibodies against either Ly5.1 or Ly5.2 diluted 1/10 (see section 2.3.1) for 1 hour on ice. The cells were washed and analysed as detailed in section 2.3.2. The lymphocytes, monocytes and granulocytes were gated on the basis of their scatter plots and analysed as separate populatiions.

(B) PCR on genomic DNA to detemine the presence of retroviral constructs

Gemonic DNA was extracted from 50µl of whole blood collected from the recipients. The nuclei were extracted from the peripheral blood cells by adding 500µl of a freshly made buffer containing 0.32M Sucrose, 10mM Tris-HCl pH 7.6, 5mM $MgCl_2$ and 1%Triton X-100; and centrifuging at 500g for 5 mins. The pellet was washed twice more in the same solution. The nuclei were resuspended in 100µl of a buffer containing 10mM Tris-HCl pH 8, 10mM EDTA, 10mM NaCl and 1mg/ml of Proteinase K and incubated at 65°C for 2 hours. These genomic DNA samples were stored at -4°C. For use in PCR reactions, 3µl of each sample was diluted in 17µl of

MQ-water and boiled for 5 mins, cooled and used in a PCR reaction as outlined in section 2.5.2.

2.11.3 Analysis of tissue samples

To analyse day 12 CFU-S (spleen colony forming units), 1-2 lethally irradiated recipients in some experiments were injected intravenously with cells equivalent to what was harvested from 1/5 of a femur. After 12 days the mice were killed and their spleens removed. Individual spleen colonies that were not in contact with other colonies were observed under a dissecting microscope while a cell suspension was extracted using a 21g needle and syringe. To determine if the colony was derived from a retrovirally infected BM repopulating cell, genomic DNA was extracted from the cells as outlined in section 2.5.3 (A) and analysed by PCR.

Recipients were killed at 7-12 months post transplantation for the analysis of tissue specimens. The spleen was removed, cut into pieces and the cells dispersed in IMDM using a loose-fitting glass homogeniser. BM cells were extracted as outined in section 2.11.1B. Cells were cytocentrifuged and Giemsa stained to observe cellular morphology. Spleen and BM cells were also washed twice in PBS and cell pellets were snap frozen in liquid nitrogen for storage at -70^{0}C. Genomic DNA was extracted from these cells (as outlined in section 2.11.2B) and analysed for the presence of retroviral DNA by PCR. Whole cell lysates were used for Western Blotting (as in sections 2.7.1) to detect retrovirus-encoded proteins.

Chapter 3: IS STRUCTURAL ALTERATION OF MYB REQUIRED FOR TRANSFORMATION ?

3.1 INTRODUCTION

De-regulated expression combined with structural alteration causes oncogenic activation of c-Myb and results in the transformation of myeloid cells *in vitro* and myeloid neoplasias *in vivo* (reviewed in Shen-0ng, 1990; Gonda, 1991 and discussed in chapter 1). For example, avian retroviruses (AMV and E26) encode activated forms of Myb that are truncated relative to c-Myb (see figure 1.2 and section 1.2.1). One study using recombinant retroviruses indicated that truncation at either terminus was sufficient for transformation of avian haemopoietic cells *in vitro*, but over-expression of full length c-Myb did not result in transformation (Grasser *et al.,* 1991). Another study indicated that truncation of c-Myb was not required for transformation of chicken neuroretinal cells (Garrido *et al.,* 1992). Structural alterations in human c-Myb have not been reported, although amplification of an otherwise apparently normal human *c-myb* gene has been detected in colon carcinoma (Alitalo *et al.,* 1984; Winqvist *et al.,* 1985) and acute myelogenous leukaemic cell lines (Pelicci *et al.,* 1984; Barletta *et al.,* 1987). However there is no evidence that increased *c-myb* expression is a causative factor in these cancers.

In the murine system, tumours and cell lines have been described in which terminally truncated c-Myb is expressed (Mushinski *et al.,* 1983; Shen-Ong *et al.,* 1984; Gonda *et al.,* 1987; Shen-Ong *et al.,* 1986; Lavu and Reddy, 1986; Weinstein *et al.,* 1987; and see section 1.3.2). *In vitro* studies of FLC infected with retroviruses encoding activated forms of *myb* have provided evidence for oncogenic activation by specific structural alterations including disruption of the negative regulatory domain (Sakura *et al.,* 1989), C-terminal truncation or mutations in the leucine zipper

structure as described in section 1.3.3 (Gonda *et al.,* 1989b, Hu *et al.,* 1991, Kanei-Ishii *et al.,* 1992).

Constitutive expression of normal c-Myb, as well as truncated forms, can block differentiation of leukaemic cell lines (Clarke *et al.,* 1988; McClinton *et al.,* 1990; Yanagisawa *et al.,* 1991; Selvakumaran *et al.,* 1992; Cuddihy *et al.,* 1993), suggesting that full length Myb, like activated forms, can inhibit differentiation of immature cells (reviewed in Gonda, 1991). However it was unclear prior to this study whether over-expression of full length c-Myb could cause transformation of primary murine haemopoietic cells and, if so, to what extent. In one study, infection of haemopoietic cells with a retrovirus carrying *c-myb* resulted in the isolation of an immature myelomonocytic cell line expressing the full length form of Myb (Gonda *et al.,* 1989a), suggesting that over-expression of full length c-Myb was able to result in immortalization. In the murine transformation experiments described above, cells over-expressing 'full length' or 'wild type' forms of Myb (with a complete C-terminus), did form colonies but at very low frequencies (Gonda *et al.,* 1989b; Hu *et al.,* 1991; Kaneii-Ishii *et al.,* 1992). However, the Myb proteins in these studies were expressed from the RED retroviral expression vector and all forms of Myb including 'full-length' lacked the first 17 N-termini amino acids of c-Myb and contained viral *gag*-encoded residues fused to the N-terminus (see also Gonda *et al.,* 1989b). Thus it is unclear whether this partial N-terminal truncation contributed to either the efficient transformation observed with the C-terminal truncated forms of Myb or the low level of transformation seen with otherwise 'wild type' Myb. In all these studies the 'transformation' of cells was assessed as the ability to block the normal differentiation of primary cells thereby maintaining cells in an immature proliferative state. The degree of transformation was measured as the number of colonies formed (in the

presence of GM-CSF) by cells expressing Myb, in comparison to normal cells that formed few or no colonies. In this chapter, experiments investigating the transforming activity of full length c-Myb in primary haemopoietic cells are described. In all experiments, transformation of full length Myb was also compared to that of CT3Myb, a C-terminally truncated mutant form of Myb, that was shown previously to contain a high transactivating capacity (Sakura *et al.,* 1989) and the ability to result in transformation of primary haemopoietic cells (Kanei-Ishii *et al.,* 1992).

RESULTS

3.2 TRANSFORMATION OF FLC USING *MYB* RETROVIRUSES

3.2.1 Generation of virus producing cell lines

The following cloning techniques were performed by Liz MacMillan (Hanson Centre for Cancer Research, Adelaide). The WT and CT3 *myb* cDNA clones inserted in p-act (Sakura *et al.,* 1989) were obtained from S. Ishii (The Institute of Physical and Chemical Research, Tukuba, Ibaraki, Japan) and subcloned into pGem3 or pGem5Z plasmids (Promega). The NcoI fragment of WT*myb* in pACT was inserted in the correct orientation into the NcoI site of pGem3 and the NcoI-XbaI fragment of CT3*myb* in pACT was inserted into the Nco1-Spe1 sites of pGem5Z. The *myb* cDNA fragments were then cloned into the pRUF(NL) retroviral expression vector (J. Rayner and T.J. Gonda unpublished- see Figure 2.1). The Nco1 fragment from pGem3-WT*myb* was cloned in the correct orientation into the Nco1 site of pRUF(NL) and the NcoI-SalI fragment from pGem5Z-CT3myb was cloned into the NcoI-XhoI sites of pRUF(NL). The RED*myb* vectors have been described previously (Hu *et al.,* 1991). The vectors, the corresponding transcripts and their protein products are shown in Figure 3.1. (Note that the 17 N-terminal residues missing from Myb proteins

expressed from the RED vectors are replaced by 28 residues encoded by the *gag* gene (Gonda *et al.*, 1989a,b.)

The RUF(CT3), RUF(WT), RED(CT3) and RED(WT) expression constructs (10μg) were co-transfected with (1μg) pSVNeo (Southern and Berg, 1982) into Ψ2 packaging cells as described in section 2.8.2. Transfected Ψ2 cells, following selection, were used as a source of *myb* retrovirus. Assessment of viral titres (as described in section 2.8.3B) was performed by Southern blot hybridization of XbaI digested DNA, which was extracted from unselected FDC-P1 cells that were infected with the *myb* viruses shown in Figure 3.2. RUF(CT3), RUF(WT), and RED(CT3) virus pools were all found to have a relative titre of 1and RED(WT) a relative titre of 0.2. A relative titre of 1 in this assay corresponds to a viral titre of $2\text{-}5x10^5$ cfu/ml using the NIH3T3 cell assay for G418R colonies (see section 2.8.3A)

3.2.2 Clonogenic Assays

Haemopoietic cells from murine foetal liver were infected with the different *myb* viruses by co-cultivation with the Ψ2 transfectants. Following infection, the FLC (FLC) were grown in liquid culture in the presence of GM-CSF for 7 days and assessed for transformation (see section 2.10.1) by their ability to form colonies in semi-solid medium. The results of colony assays from 27 different experiments are compiled in Figure 3.3, and show that FLC infected with RUF*myb* constructs formed comparable numbers of colonies to those infected with the corresponding RED*myb* constructs.

3.2.3 Is Amino-terminal truncation of Myb is required for transformation?

As stated above, the Myb proteins expressed from the RED vector have an amino-terminal truncation of 17 amino acids, while those from the RUF vector have a complete amino terminus. Since infection with each of the four *myb* viruses generated

substantial numbers of colonies, it can be concluded that amino-terminal truncation is not necessary for transformation of FLC by Myb. Quantitative comparison of transformation by RED(WT) and RUF(WT) was not possible since the viral titre (determined by infection of FDC-P1 cells followed by Southern blotting) of the RED(WT) producer cells used was $^1/_5$ of that of RUF(WT) as shown in Figure 3.2. However, transformation of FLC by RED(CT3) could be directly compared to RUF(CT3) since the titres of these viruses were equivalent (Figure 3.2). The results shown in Figure 3.3 indicate that FLC infected with RUF(CT3) formed ~~slightly~~ more colonies, on average, than those infected with RED(CT3), indicating that the 17aa N-terminal truncation did not enhance transformation by CT3Myb.

3.2.4 Full length c-Myb over-expressed in FLC leads to their transformation

The second major feature of the data shown in Figure 3.3 is that the FLC infected with the RUF(WT) *myb* virus formed high numbers of colonies, indicating that enforced expression of WT (i.e. full length) Myb is sufficient for the transformation* of these cells. Colony formation following infection with RUF(WT) and RUF(CT3) could be directly compared since the viral titres for these constructs were equivalent (Figure 3.2). Statistical analysis of the numbers of colonies obtained for RUFWTMyb and RUFCT3Myb infected FLC in 27 separate experiments using the paired T test revealed that CT3Myb gave rise to significantly more colonies ($p<0.008$). The median of the ratios of colonies formed by CT3 compared to WT for all of the experiments was 1.8. Thus the full length, proto-oncogenic form of c-Myb, when continuously over-expressed, was able to transform FLC *in vitro,* but the degree of transformation was significantly lower than that seen with CT3Myb.

3.2.5 Detection of expression of retroviral Myb proteins

To determine whether these results were influenced by differences in the levels

* see additional notes

of expression of the various constructs, Western blot analysis of Myb proteins extracted from the infected FLC was conducted using mAb 5.1, as described in section 2.7.1. The expected virally encoded Myb proteins of 38, 75, 39 and 76 kD for RUFCT3, RUFWT, REDCT3 and REDWT respectively, were expressed at similar levels as indicated in Figure 3.4.

3.3 CHARACTERISTICS OF MYB TRANSFORMED FLC.

3.3.1 Surface Marker Analysis

Flow cytometric analysis of surface marker expression by WT*myb* and CT3*myb* infected FLC at 2 weeks post infection showed no differences between the two populations. Both were Thy1$^-$, Mac1$^+$, Gr1$^+$, c-Kit$^-$ (Figure 3.5A), indicating that the cells transformed by WTMyb were of the same lineage as those transformed by CT3Myb. With prolonged growth in liquid culture, the CT3Myb transformed FLC became heterogeneous and some Thy1$^+$ cells as well as c-Kit$^+$ cells could be detected as shown in Figure 3.5B, which is in agreement with other studies of more established lines (Gonda *et al.,* 1989a, Gonda *et al.,* 1993). WTMyb transformed FLC did not survive ~~up~~ to this time point due to a relatively high tendency to differentiate during long term culture. The presence of Gr1 and Mac1 markers indicate that the Myb transformed FLC are of the myeloid lineage and the absence of the c-Kit and Thy1 markers indicate that at early times post-infection, the majority of FLC have a relatively mature phenotype. The emergence of c-Kit$^+$,Thy1$^+$ cell lines implies that immature cells were present in the initial cultures and had a growth advantage over the other cells.

3.3.2 Cellular characteristics

Morphological analysis of infected FLC at 9 days post-infection, as described

in section 2.2.2, is shown in Figure 3.6. The micrographs indicate that CT3*myb* transformed FLC retained immature characteristics in contrast to uninfected FLC cultures where the surviving cells had differentiated into macrophages and neutrophils. WTMyb-expressing FLC were a mixed population with some showing similar characteristics to those expressing CT3Myb, and other cells showing more differentiated features.

3.3.3 Colony morphology

Examples of the various types of colonies formed by *myb*-infected FLC in methylcellulose are shown in Figure 3.7. Using the criteria previously applied to the analysis of differentiation of myeloid leukaemias (Metcalf and Nicola, 1982) the compact colonies are suggestive of proliferating, undifferentiated cells and the diffuse and "mixed" colonies are representative of proliferating, partially differentiated cells. In a typical experiment, 71.6 ± 4.9% of the total number of colonies expressing RUF(WT), were diffuse or mixed, whereas only 46.7 ± 2.9% of the colonies expressing RUF(CT3) were diffuse or mixed. Taken together the data indicate that WTMyb like CT3Myb can enforce a proliferative state, but WTMyb transformed cells have a greater propensity to differentiate.

3.4 EFFECT OF CELL DENSITY ON TRANSFORMATION BY *MYB*

During the course of these experiments, it was observed that the number of FLC used during co-cultivation (which determined the cell density in liquid culture post infection) appeared to affect the number of colonies formed per 2,000 cells plated in the transformation assay. Indeed, analysis of the data of Figure 3.3 showed a clear trend towards increasing transformation by WTMyb relative to CT3Myb with increasing cell density (data not shown). To further examine this phenomenon,

experiments were conducted in which the FLC were cultured for 7 days at various densities after infection with RUF(WT) or RUF(CT3), following which transformation was assessed in a standard colony assay. Figure 3.8 shows the results of a typical experiment; similar results were observed in three separate experiments. From low density cultures, the colony yields (per $2x10^4$ cells plated) were minimal and steadily increased with increasing cell density. At very high densities the cultures became overgrown, which resulted in cell death, and hence low colony counts. Futhermore, the effect of cell density on transformation varied with the *myb* construct used to infect the FLC. CT3Myb-infected FLC showed greater levels of transformation (colony formation) at lower cell densities, and a higher peak level of transformation when compared to WTMyb expressing FLC.

3.4.1 Presence of growth factor(s) produced by MTHC can increase transformation at low densities.

It was of interest to determine whether the effect of cell density on transformation was due to the effects of a previously identified autocrine factor produced by *myb*-transformed myeloid cells, that is known to enhance their proliferation (MacMillan and Gonda, 1994). Figure 3.9 shows the results of a standard transformation experiment conducted with or without the addition of conditioned medium from high density cultures of Myb transformed cells, to FLC cultured for 7 days post infection at high or low densities. This experiment was performed by Elizabeth Macmillan and is reproduced in this thesis with her permission. The data indicate that transformation of WT*myb* infected cells cultured at low density could be increased by the presence of conditioned medium. In this experiment, there was no significant effect of conditioned medium on transformation by CT3Myb which may

be because at "low density" transformation was already close to maximal. At high density there was no significant effect on transformation by either virus.

Figure 3.1: Structure and expression of RED(myb) and RUF(myb) vectors.

Part A of each panel illustrates the structure of the plasmids containing the RED(myb) and RUF(myb) retroviral expression vectors (as indicated). The region between (and including) the long terminal repeats (LTR) represents viral sequence except for the region labelled "myb" which represents a murine cDNA clone of *myb* inserted into the vector.

Likewise, in each case part B illustrates the structure of spliced and unspliced viral RNAs initiated from the 5'LTR. Part C shows the protein encoded by the spliced mRNA expressed from each of the two vectors.

The Myb proteins expressed from RED lack the first 17 amino acids of c-Myb and instead contain 28 residues encoded by viral *gag* sequences; the Myb proteins expressed from RUF contain the natural c-Myb N-terminus.

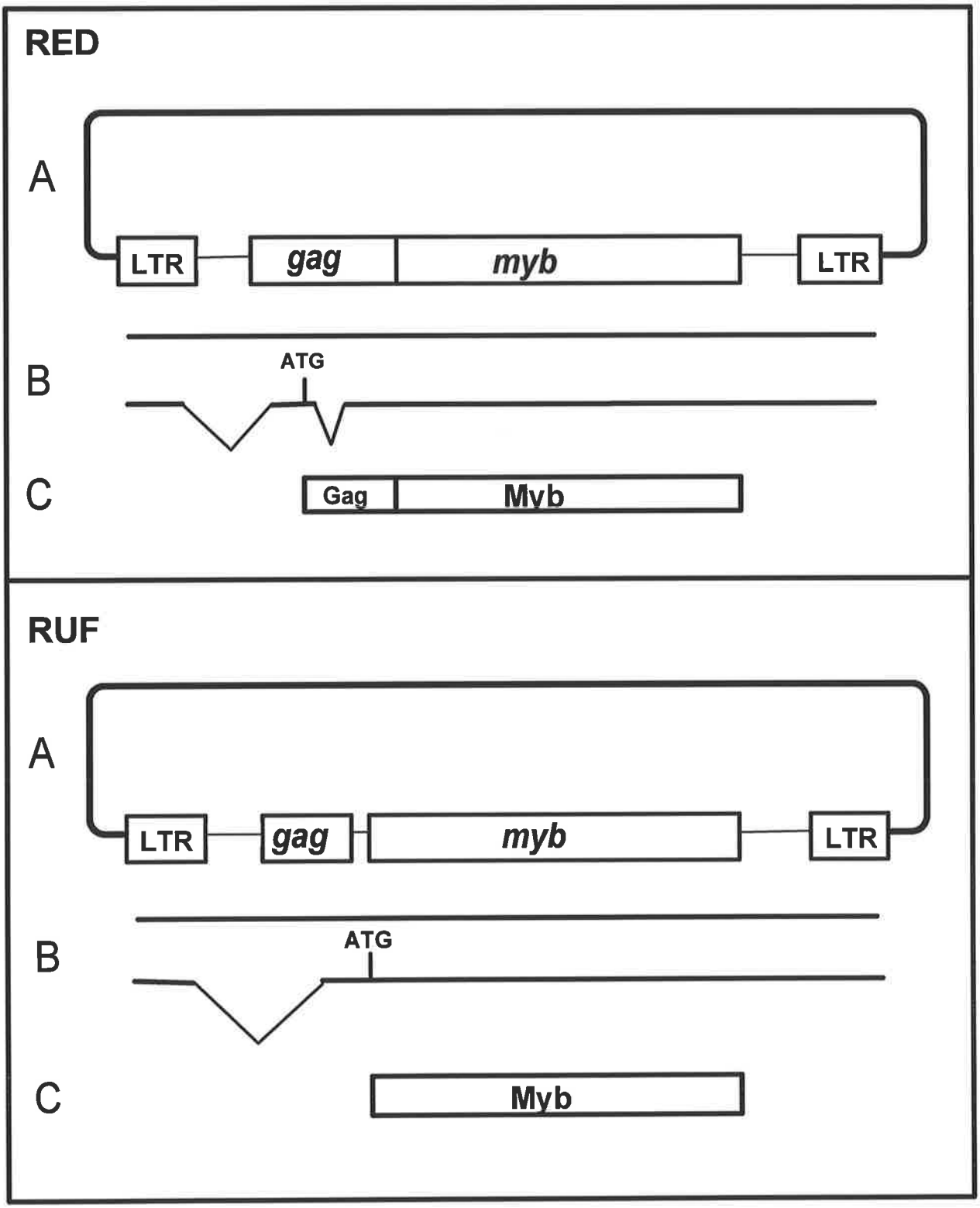

RED
A
LTR
gag
myb
LTR
B
ATG
C
Gag
Myb
RUF
A
LTR
gag
myb
LTR
B
ATG
C
Myb

Figure 3.2: Relative titres of the RUF(myb) and RED(myb) viruses used in transformation assays.

The photograph shows Southern blots of genomic DNA extracted from unselected FDC-P1 cells following infection with the indicated *myb* viruses as outlined in section 2.8.3B. The DNA samples were digested with Xba1 and hybridised with a *myb* probe as specified in more detail in section 2.5.4. The sizes, in kb, of marker DNA fragments are shown on the right. The endogenous *myb* bands of 3.3kb are indicated by an asterisk (*) and the proviral bands for the various constructs are indicated by arrows (>). The relative titre is reflected by the ratio of intensity of the proviral band to that of the endogenous c-*myb* bands assessed by a Phosphor-Imager. The relative titres were equivalent for the RUF(WT), RUF(CT3) and RED(CT3) virus pools of 1, but the titre for RED(WT) was $^1/_5$.

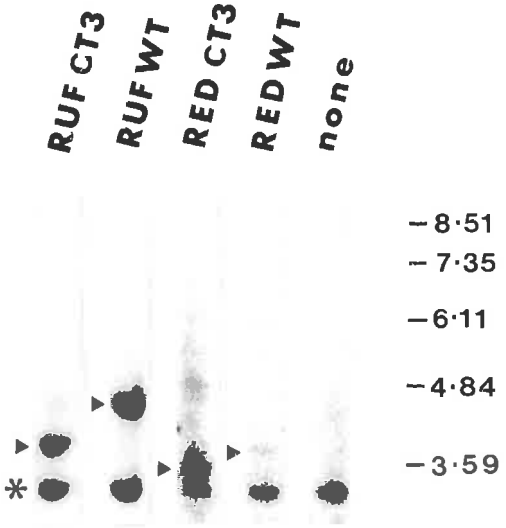

RUF CT3
RUF WT
RED CT3
RED WT
none
8·51
7·35
6·11
4·84
3·59

Figure 3.3: Transforming activity of WTMyb and CT3Myb encoded by RUF(myb) and RED(myb) expression constucts in FLC.

The figure shows results of the colony assays from a series of experiments in which FLC were infected with the indicated RUF(myb) and RED(myb) viruses as detailed in section 2.10; uninfected FLC are indicated by "none". FLC infected with the various *myb* viruses were cultured in GM-CSF for 7 days and then plated in methylcellulose at $2x10^4$/plate in all cases. The symbols represent the average number of colonies formed by the FLC in each experiment. The horizontal lines represent the mean number of colonies averaged over all the experiments for each of the different constructs.

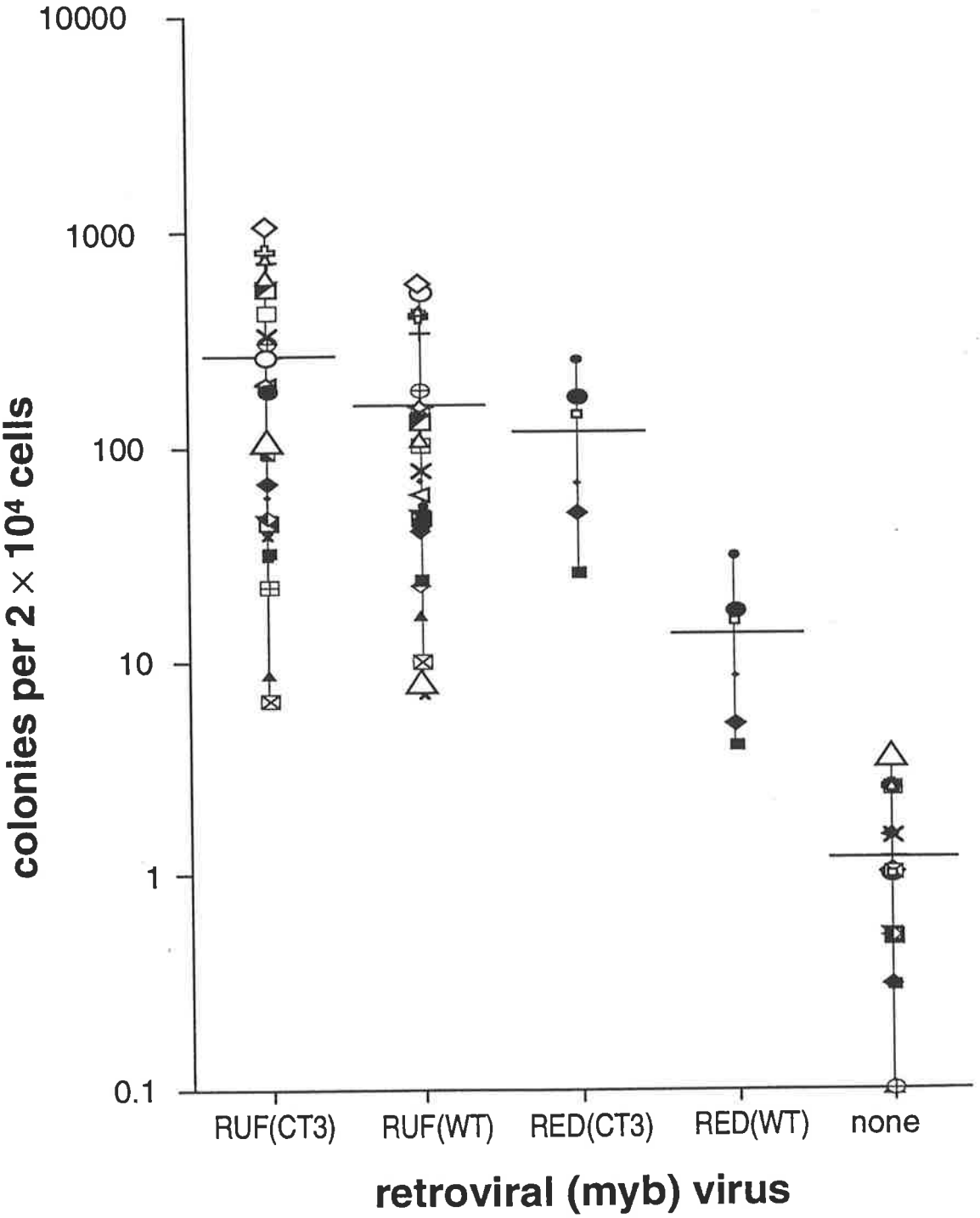

10000
1000
100
10
1
0.1
colonies per 2 × 10⁴ cells
RUF(CT3)
RUF(WT)
RED(CT3)
RED(WT)
none
retroviral (myb) virus

Figure 3.4: Immunodetection of Myb proteins expressed by transformed FLC.

The photograph shows western blots of whole cell lysates from FLC which had been transformed by the indicated *myb* viruses, probed with the anti-Myb mAb 5.1 as described in section 2.7.1. The arrows indicate the WTMyb and CT3Myb proteins expressed from the retroviral constructs.

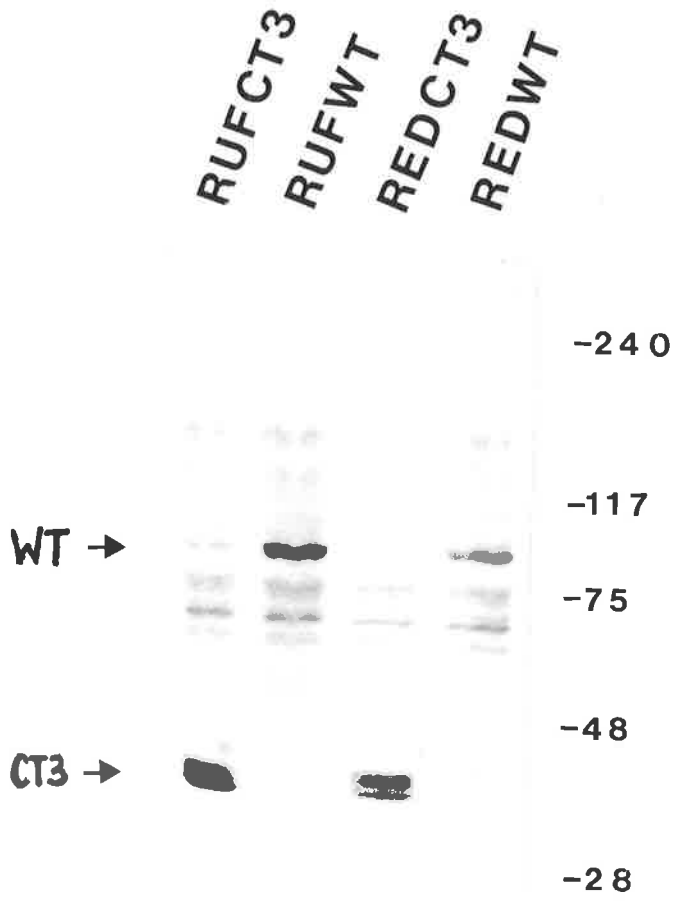

RUFCT3
RUFWT
REDCT3
REDWT
-240
-117
-75
-48
-28
WT
CT3

Figure 3.5: Surface marker expression analysis of transformed FLC.

The graphs show the fluoresence histograms of the FLC stained with antibodies for the various surface markers at 2 weeks (A) or 2 months (B) post infection in culture containing GM-CSF. Immunofluorescence was carried out as described in section 2.3.2 using antibodies 30H12 (anti-Thy1), ACK-2 (anti-murine c-Kit), 8C5 (anti-Gr1) and M1/70 (anti-Mac-1). C- represents the negative control of no primary antibody.

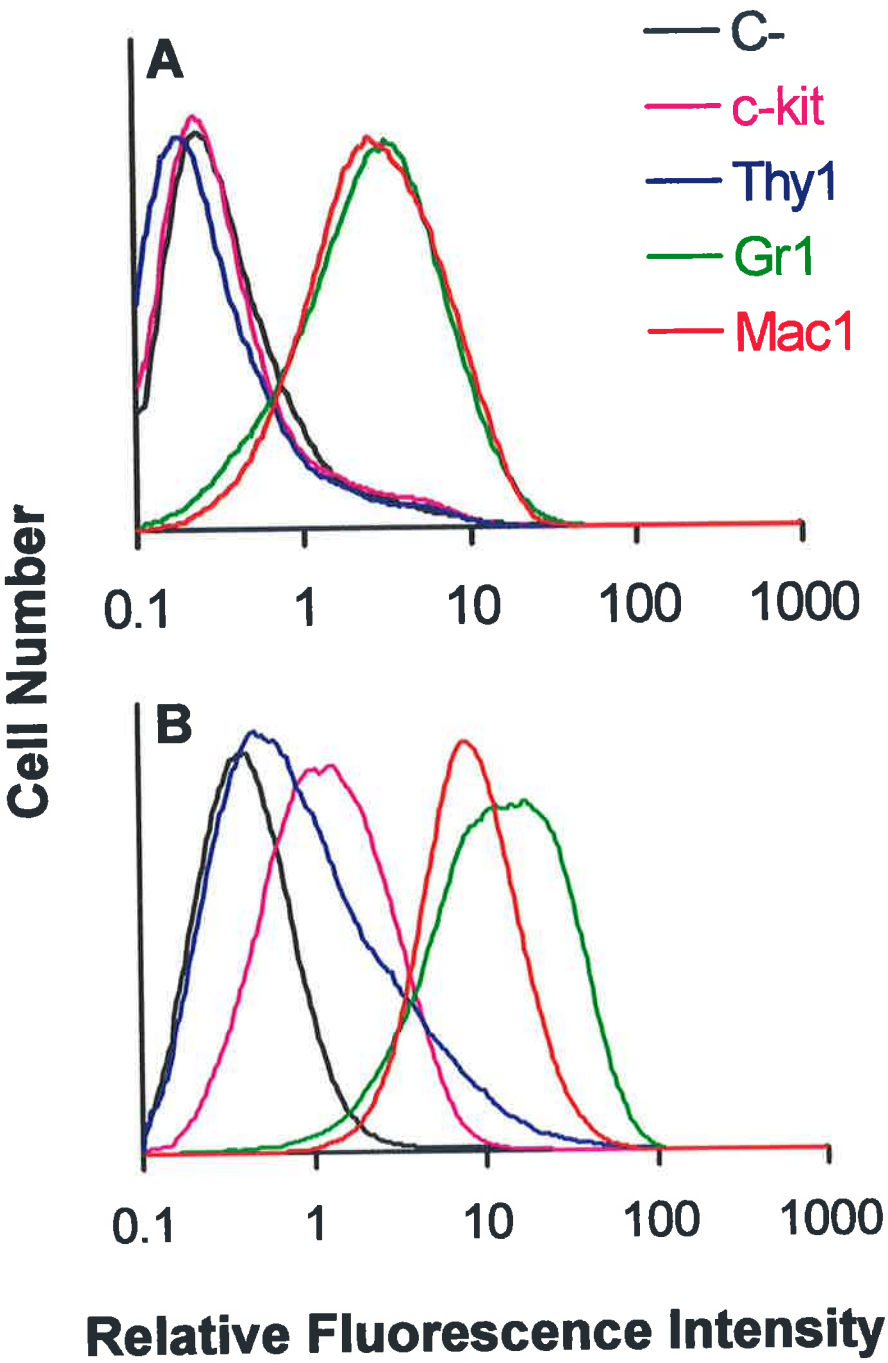
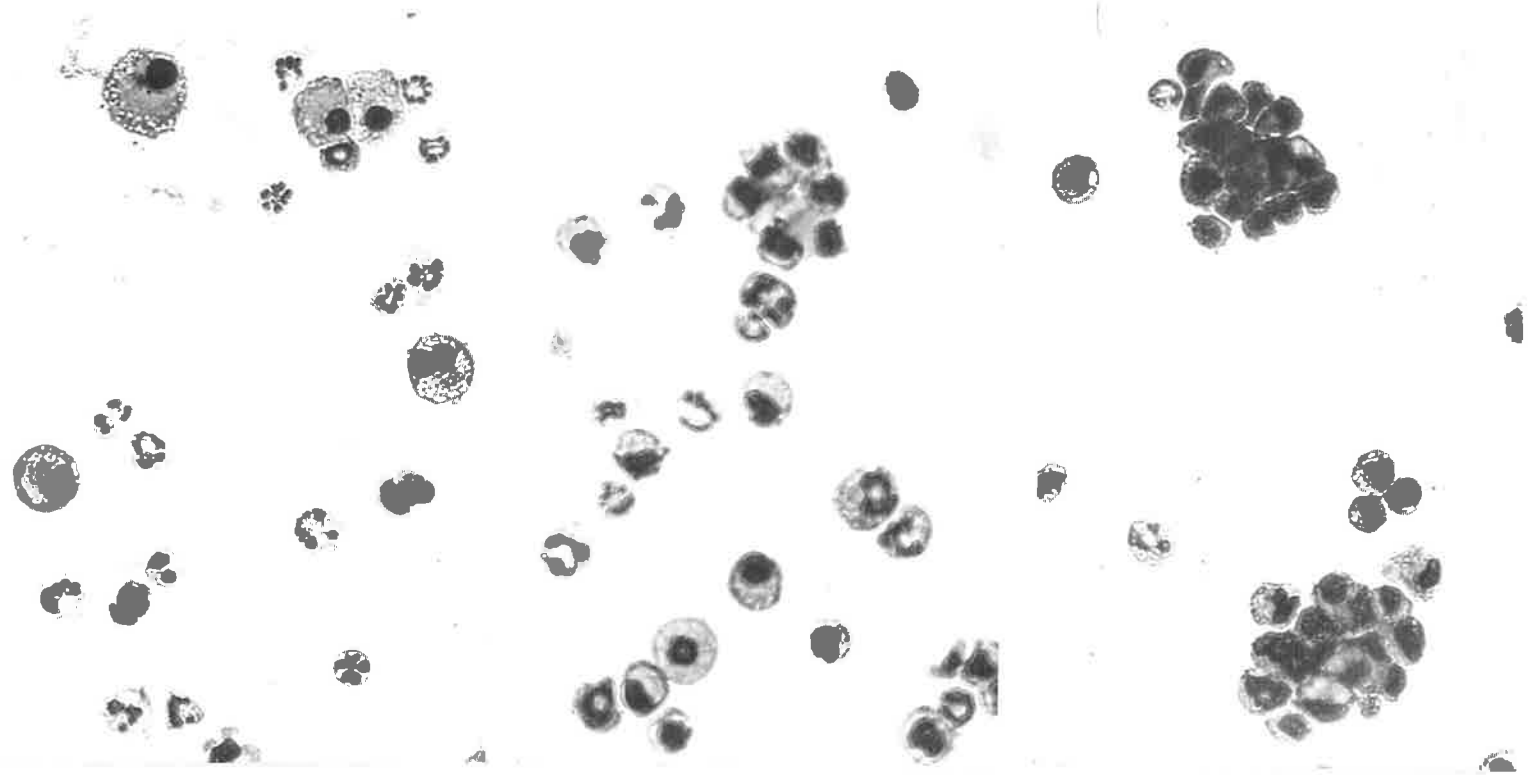

A
C-
c-kit
Thy1
Gr1
Mac1
0.1
1
10
100
1000
B
0.1
1
10
100
1000
Cell Number
Relative Fluorescence Intensity

Figure 3.6: Phenotype of *myb* transformed FLC.

The micrographs show cytocentrifuge preparartions of FLC at 9 days following infection with the indicated RUF(*myb*) viruses stained with Wright-Giemsa (as described in section 2.2.2). **The cells shown were photographed at an initial magnification of 40x.**



(a) none (b) WT Myb (c) CT3 Myb

Figure 3.7: Morphology of colonies formed by Myb transformed FLC.

The micrographs show examples of the different types of colonies formed in methylcellulose by RUF(*myb*)-infected FLC plated 7 days post infection. The colonies were classified as (A) "compact" i.e. rounded, well defined type with cells in close contact; (B) "diffuse" i.e. spread out colonies with cells not in contact; or (C) "mixed" i.e. diffuse colonies with compact centres.

The colonies shown were photographed at an initial magnification of 10x.

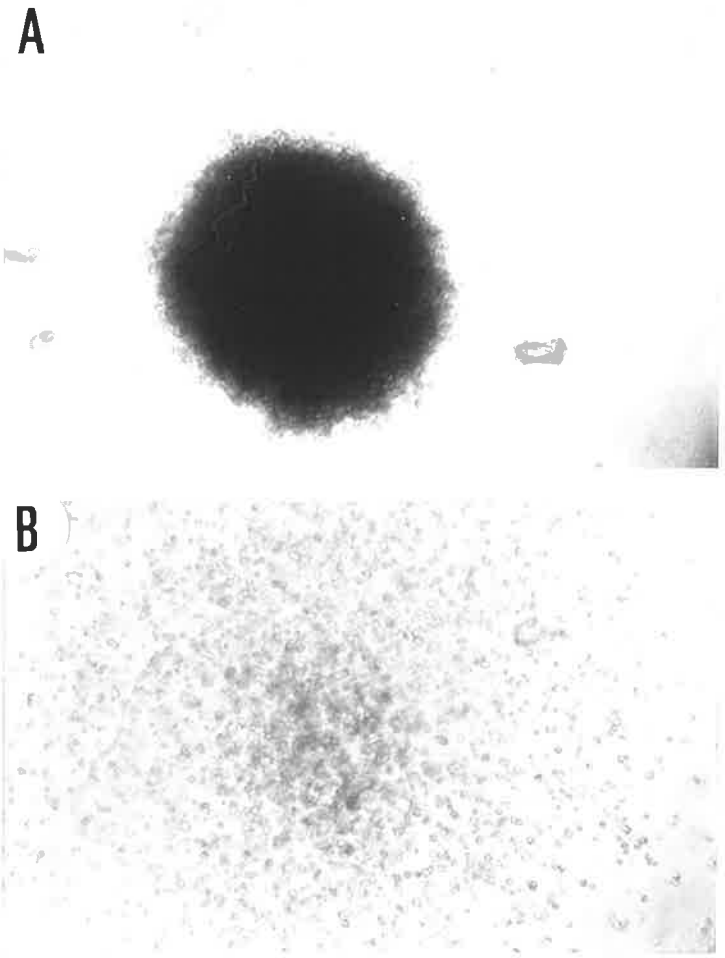

A
B
C

Figure 3.8: Transformation Assay of *myb*-infected FLC cultured at various cell densities.

The graph shows the results of an experiment (carried out as described in section 2.10.1) in which 10^6 FLC were co-cultivated with Ψ2 cells producing either WT or CT3 RUF*myb* viruses as indicated. "None" indicates uninfected FLC that were co-cultivated with untransfected Ψ2 cells. Following infection, FLC cultures were initiated at the cell densities indicated on the graph. After 7 days, $2x10^4$ cells were plated in a standard colony assay. Each point represents the number of colonies formed in an individual culture.

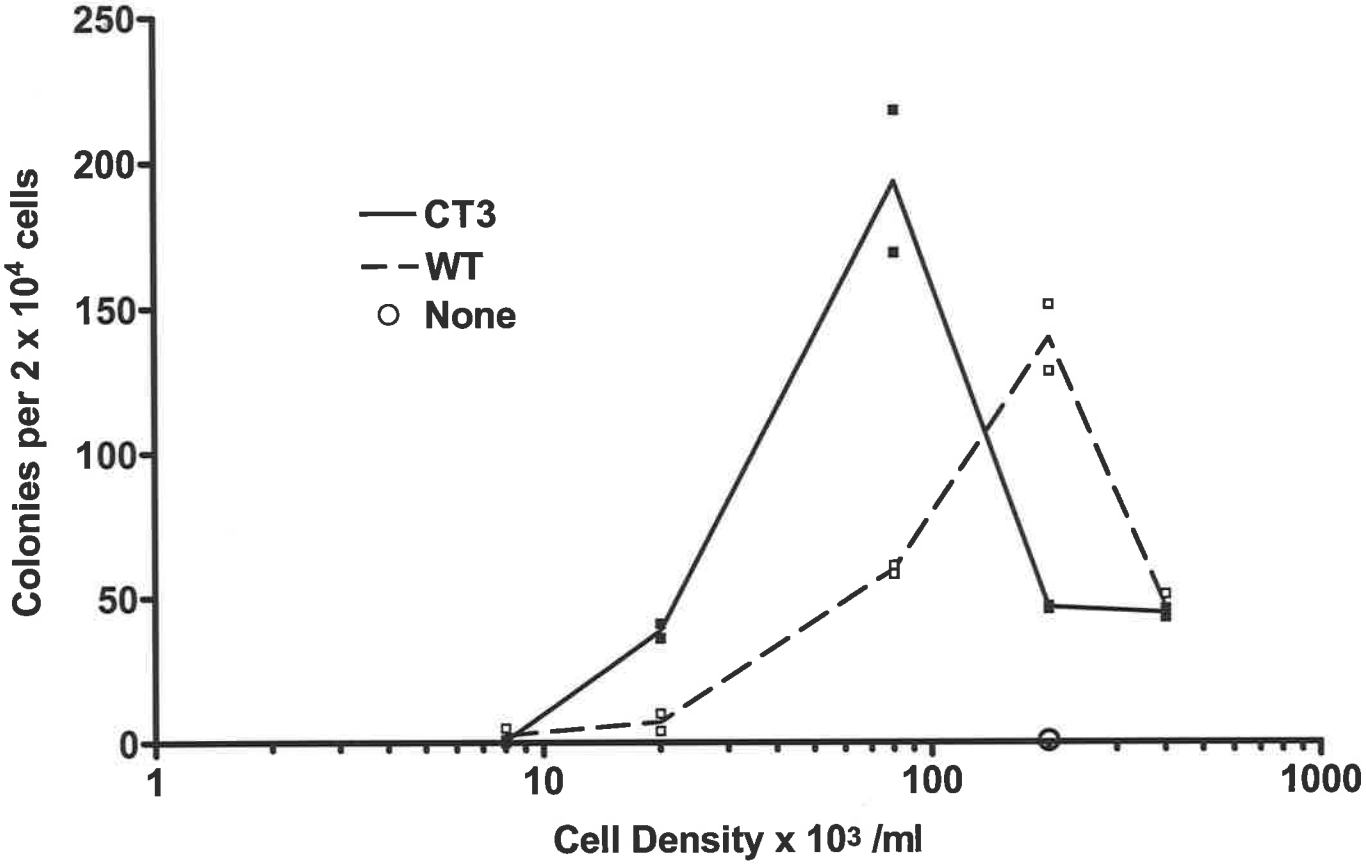
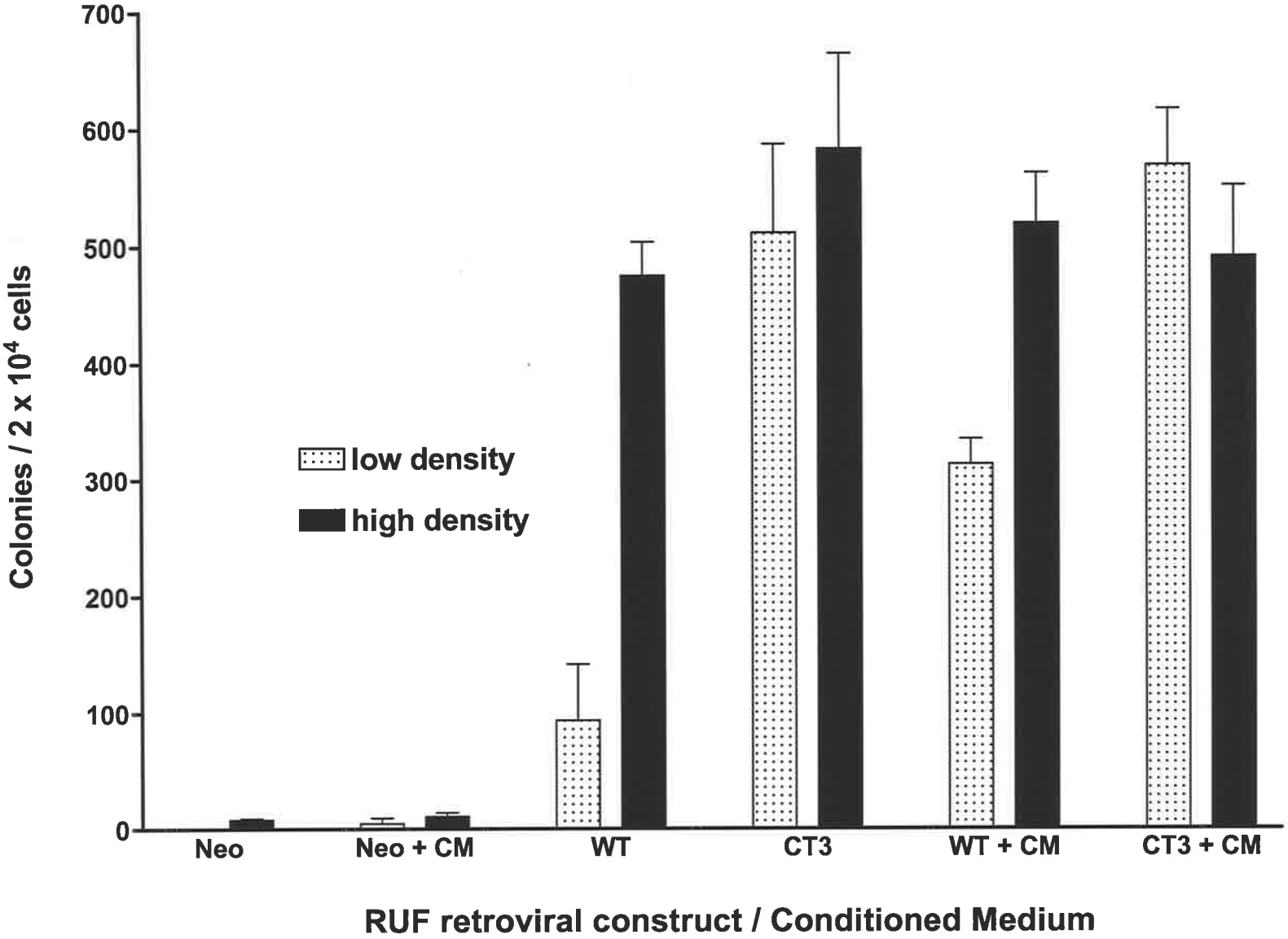

250
200
150
100
50
0
Colonies per 2 x 10⁴ cells
CT3
WT
None
1
10
100
1000
Cell Density x 10³ /ml

Figure 3.9: Transformation assay of Myb transformed FLC in the presence of conditioned medium.

The figure shows the results of an experiment (carried out by Elizabeth MacMillan as described in section 2.10.1) in which FLC with a control RUFneo retrovirus or with WT or CT3 RUF(myb) retroviruses, as indicated and cultured in suspension either in the absence or presence of 50% (v/v) conditioned medium (CM) from CT3Myb transformed cells. The infected FLC were seeded either at 2 x 10^4 cells/ml (low density) or 2 x 10^5 cells/ml (high density) during the 7 day culture period prior to plating. The number of colonies generated from 2 x 10^4 cells/ml after 7 days in methylcellulose are shown as the average +/- standard deviation of duplicate cultures.


700
600
500
400
300
200
100
0
Colonies / 2 x 10^4 cells
low density
high density
Neo
Neo + CM
WT
CT3
WT + CM
CT3 + CM
RUF retroviral construct / Conditioned Medium

3.5 DISCUSSION

It has been shown here that enforced over-expression of WTMyb in haemopoietic cells leads to their transformation *in vitro*. FLC infected with WTMyb virus, like those infected with CT3Myb virus, were transformed into a highly proliferative state in the presence of GM-CSF, as indicated by their sustained clonogenicity (Figure 3.3). In agreement with previous studies (Gonda *et al.*, 1989b, Hu *et al.*, 1991) transformation by CT3Myb was more efficient than transformation by WTMyb (Figure 3.3) confirming that structural alterations, such as C-terminal truncation, can significantly enhance the transforming capacity of c-Myb. However, the extent of transformation by WTMyb relative to CT3Myb was considerably greater than that seen in earlier studies; this issue is addressed further below.

The morphology of the WTMyb-infected cells (Figure 3.6), the higher proportion of diffuse colonies formed by these cells and the inability to give rise to long term cell lines, all indicated that over-expression of c-Myb was unable to completely block the differentiation of the transformed cells, and was less efficient in doing so than CT3Myb. Additionally, surface marker analysis (Figure 3.5), indicated that these cells initially lacked markers of early immature cells (c-Kit and Thy1), but did express the granulocyte-macrophage lineage markers (Gr1 and Mac1). However, we observed an increase in expression of c-Kit and Thy1 on CT3Myb transformed FLC, concommitant with a decrease in morphologically differentiated cells during prolonged culture. This suggests that the cells with immature myeloid phenotype were selected during long term culture. It has been shown previously that expression of early markers c-Kit and Thy1 on *myb*-transformed cells is heterogeneous and is modulated by the growth factors to which they are exposed (Gonda *et al.*, 1993).

It was also observed that transformation by *myb* was affected by the density at which cells were cultured immediately following infection (Figure 3.8). This effect may have been responsible for much of the variation observed between the individual experiments (Figure 3.3) as the number of FLC used for each infection, which subsequently determined the cell density during liquid culture, was varied between many of these experiments depending on the numbers of FLC available. Furthermore, since truncated *myb* seems to be more capable of transforming cells under sub-optimal conditions these results may explain the inefficiency of transformation by WTMyb in previous studies (Gonda *et al.,* 1989b; Hu *et al.,* 1991; Kaneii-Ishii *et al.,* 1992). The influence of cell density and other external factors may also explain the differences observed in transformation by WTMyb in previous murine studies (Gonda *et al.,* 1989b; Hu *et al.,* 1991; Kaneii-Ishii *et al.,* 1992) and those reported here. Similarly in the avian system, early studies showed that expression of full length c-Myb did not result in transformation (Grasser *et al.,* 1991) in contrast to a recent report showing that avian c-Myb is able to cause transformation of monocytic and granulocytic cells (Fu and Lipsick, 1997).

The mechanism for the density effect observed was further investigated. The decrease in colony numbers observed at high densities (Figure 3.8), was almost certainly due to the over-growth of the FLC and subsequent cell death. However the reasons for the increase in colony numbers with increasing density are potentially more important. The increase in transformation of WT*myb* infected FLC cultured at low density with the addition of conditioned medium was almost certainly due to the effects of growth factors produced by Myb transformed cells. The relevant factor(s) may include the autocrine activity, produced by *myb*-transformed myeloid cells, that is known to enhance their proliferation (MacMillan and Gonda, 1994). CSF-1 may

also play a role as *myb*-transformed cells both produce this factor and respond to it (Gonda *et al.,* 1993; Macmillan and Gonda 1994). However, for WTMyb, the level of transformation obtained by the addition of conditioned medium at low density was still lower than that observed at high density. This may be due to cell proximity effects at high density resulting in higher local concentrations of growth factors. Furthermore, by analogy to the effect of fibroblast feeder layers (MacMillan and Gonda, 1994), it could be that normal FLC or their differentiated progeny, which are present at high density during the culture period, produce factors that support the growth of *myb*-infected FLC.

The results in Figure 3.8 also reveal an important difference in the behaviour of cells infected with WTMyb and CT3Myb viruses. At lower cell densities, CT3Myb infected cells generated substantially more colonies than WTMyb infected cells even in the absence of conditioned medium. A possible explanation for this difference could be that CT3Myb expressing cells produce higher levels of the relevant growth factor(s) or, alternatively, that they are more sensitive to autocrine or paracrine factors. In any case, these data suggest an interaction between the effects of growth factors and the effects of Myb on the proliferative state of Myb expressing cells. Thus, the environment of *myb*-expressing cells immediately post infection appeared to influence the ability of *myb* to transform haemopoietic cells and this influence varied with the form of Myb involved.

Taken together, these results demonstrate that WTMyb, under appropriate conditions, is capable of transforming primary haemopoietic cells *in vitro* as indicated by continuous proliferation and partially blocked differentiation. However, further structural alterations that activate c-Myb by disrupting the negative regulatory domain appear to enhance transformation and to cause a more complete block in

differentiation. It will now be important to establish the effects of continuous over-expression of WT and activated c-*myb* *in vivo* using an animal model system, particularly since our results suggest that the environment of Myb expressing cells can influence their transformation. The results would be relevant in terms of indicating whether gene amplification of c-*myb* observed in some human tumours may be involved in the initiation of the disease state and whether regulatory defects leading to continuous expression of Myb could contribute to leukaemias or other tumours.

Chapter 4. OVER-EXPRESSION OF ACTIVATED MYB *IN VIVO*

4.1 INTRODUCTION

The effects of over-expression of activated Myb on haemopoietic cells *in vitro* has been well researched. The effects of over-expression of Myb on the haemopoietic system *in vivo* has not been addressed to the same extent.

In the murine system, as detailed in Chapter 3, transformed cells have clonogenic potential and are able to give rise to long term cell lines only in the presence of added growth factor, and these lines are not tumourigenic unless they aquire factor-independence (Gonda *et al.,* 1989a). Studies with mice involving insertional mutagenesis of c-*myb* by Mo-MLV, revealed that although activated forms of the *gag-myb* fusion (associated with MML) was expressed in the spleen and BM of mice, they did not develop MML (Nason-Burchenal *et al.,* 1993). However, in the same experiments mice infected with Mo-MLV and injected with pristane, developed MML in 50% of cases suggesting that Myb is an early step in murine leukaemia (Nason-Burchenal *et al.,* 1993). Similarly, another experiment showed that activated c-myb due to proviral integration following MoMLV infection was detectable in pre-leukaemic mice (Belli *et al.,* 1995). Most of these experiments address the effects of Myb *in vivo* following infection with viruses, which relies on systemic spread resulting in infection of haemopoietc cells.

In order to obtain expression specifically and only in haemopoietic cells, the experiments in this chapter were based on the reconstitution of the haemopoietic system of mice with cells expressing Myb. This procedure was established by Williams *et al.,* (1984), and since then has been used to determine the effects of other oncogenes on the haemopoietic system as well as to establish models for leukaemogenesis (reviewed by Bonham and Symonds, 1992). The system is based on

the eradication of the haemopoietic system of recipient mice by lethal irradiation, followed by transplantation of genetically manipulated immature BM cells from donor mice. These reconstituted recipient mice are then assessed for the specific effects of the genetic manipulation on the haemopoietic system. Myeloproliferative disorders and leukaemias heve resulted from the reconstitution by BM cells expressing various oncogenes including transcription factors e.g. v-*myc* (Bonham *et al.,* 1992) and E2A (Kamps and Baltimore, 1993); protein kinases e.g. v-*abl* (Chung *et al.,* 1991), growth factors e.g. GM-CSF (Johnson *et al.,* 1989); and receptors e.g. v-*erbB* (von Ruden *et al.,* 1992), v-*fms* (Heard *et al.,* 1987) and PDGF-R (Yan *et al.,* 1994).

In contrast to the earlier studies mentioned above, this procedure offered advantages including the specific expression in haemopoietic cells and the absence of systemic virus infection. This system is based on the introduction of the oncogene into early repopulating cells, which give rise to the blood cells, thereby providing the ability to assess the effects of the oncogene in any of the haemopoietic blood cell lineages.

To analyse the transforming effects of Myb *in vivo*, CT3Myb, one of the most oncogenic mutant forms *in vitro* (Kaneii-Ishii *et al.,* 1992), was introduced into donor BM cells by retroviral infection. As a negative control for the experiments the vector alone (pRUFneo) was also introduced. The donor cells were used to repopulate lethally irradiaited recipient mice, that were analysed over a period of up to 12 months.

4.2 OPTIMISATION OF THE TRANSPLANTATION PROCEDURE

A schematic flow diagram of the complete procedure is shown in Figure 4.1

and described in section 2.11.1. In order to optimise the procedure for analysis of the effects of Myb, it was important to obtain the highest level of infection of donor BM cells.

4.2.1 Optimal method of infection

To assess the best method of infection, BM cells obtained from 5FU treated mice were infected via two different methods (co-cultivation with virus producing cells or culture in supernatant from virus producing cells). Treatment of donor mice with 5FU is now a standard part of the protocol, since it was shown to be required for effeicient gene transfer and to be beneficial in retaining the repopulating activity of stem cells (Bodine *et al.,* 1991). Co-cultivation with Ψ2 virus producers was performed essentially as described in section 2.8.3 (B), where cells from one femur were co-cultivated for 3 days with $1x10^6$ Ψ2 producers as shown in Figure 4.1. Concurrently, cells were infected by viral supernatant as described in section 2.8.3 (A), with all other parameters the same as for co-cultivation. The infected cell populations were not injected into recipient mice, but were assessed for transformation by colony assay. This was carried out as for the transformation assay of FLC described in section 2.10 (C), except that the cells were cultured for 10 days prior to plating. Colony counts from 3 different experiments are shown in Table 4.1. As the number of colonies represents the number of cells transformed by Myb, the results indicate that co-cultivation is the better method for infection in order to analyse transformation *in vivo*.

4.2.2 Assessment of reconstitution

A preliminary experiment was conducted to analyse successful reconstitution of recipients with donor BM as detailed in section 2.11.1. BM cells from male donor mice were infected by co-cultivation with either CT3myb virus or virus carrying

vector only (RUFneo) in culture medium containing IL-3, IL-6 and SCF, a combination of growth factors that was shown to be beneficial for efficient retroviral mediated gene transfer into murine haemopoietic cells (Bodine *et al.,* 1989; Luskey *et al.,* 1992) as well as for the maintenance in culture of stem cells with repopulating ability (Szilvassy and Cory, 1994). The BM cells were injected into lethally irradiated female recipient mice. Peripheral blood samples from the recipients were analysed (see section 2.11.2) for donor derived cells at various time points following transplantation. Genomic DNA was extracted as described in section 2.11.2 (B) and assayed by PCR for the Y-specific Zfy gene (Figure 4.2A). The results shown in Figure 4.2B indicate that reconstitution of recipient mice with cultured donor BM was successful in most cases. However, from this method it was not possible to quantitate the level of donor cells, or to determine if all lineages were derived from donor cells.

All the following experiments were conducted using congenic donor mice as shown in Figure 4.1 and detailed in 2.11.1 (A). To detect the presence of donor cells, peripheral blood cells from recipient mice were assessed for the expression of the Ly5.1 and Ly5.2 markers by immunofluoresence and flow cytometry as outlined in section 2.11.2 (A). Examples of the fluoresence profiles and the calculated results from analysis of preipheral blood from recipients at various time points are shown in Figure 4.3 and Table 4.2. The representative examples in Figure 4.3 show that the positive and negative cells are clearly distinguishable. The analysed data in Table 4.2 show the average percentage positive of the three gated populations, since the percentage positive cells were very similar for the different lineages in all cases. The data indicate that all recipients showed donor cell reconstitution of the haemopoietic system. In most experiments a few recipient mice died over the time course. This was not an effect due to Myb, since death was also observed in control mice that were

transplanted with 'Neo infected' BM. Autopsy of the mice showed that the spleens of these mice were extremely underweight suggesting death possibly due to lack of blood cells, caused by limiting numbers of long term repopulating cells.

4.2.3 Detection of retroviral *myb*

To determine if recipient mice were reconstituted with infected BM, genomic DNA from the peripheral blood of recipient mice was analysed by PCR for retroviral *neo* and *myb* as described in sections 2.11.2B. As an example, Figure 4.4 (A) shows the amplified *myb* PCR products from all the samples analysed at a single time point (5 months post transplantation) in one experiment. The PCR products were myb specific, as shown by Southern Blot analysis in Figure 4.4 (B). The product amplified from the endogenous *myb* gene acts as a control for the genomic DNA in each sample. The results show that the majority of recipients were reconstituted with '*myb*' infected BM and these cells were able to give rise to blood cells. Similarly, most of the recipients transplanted with '*neo*' infected BM had the *neo* sequence in the peripheral blood, as shown in Figure 4.4 (C). This indicated that the procedure used was successful in producing recipient mice displaying long term reconstitution with retrovirally infected donor BM cells. However, some recipients did not have detectable *myb* or *neo* infected blood cells suggesting that no repopulating cells were infected prior to transplantation or that infected repopulating cells did not successfully repopulate the recipients.

The CFU-S assay has been used previously to assess retroviral infection of repopulating haemopoietic cells. Although the cells that give rise to the CFU-S have been shown to be distinct from the 'long-term repopulating' cells, they are accepted as being direct descendants of 'long-term repopulating' cells (Jones *et al.,* 1990; Ploemacher *et al.,* 1989). Therefore, the CFU-S forming cell is the 'earliest'

repopulating cell for which an *in vivo* short term assay exists to determine the degree of infection of 'long-term repopulating' cells. To estimate the proportion of long term repopulating cells that were infected with retrovirus, the proportion of day 12 CFU-S that were positive for the presence of retroviral DNA was analysed. Recipient mice to be used for the CFU-S assay were injected with BM cells following infection that were equivalent to those derived from 1/5 of a donor femur, in comparison to long term recipients that were injected with cells equivalent to one femur. At 12 days after injection, the mice were killed and the the spleen colonies analysed by PCR for *myb* as described in section 2.11.3. Figure 4.5 gives an example of the results obtained in one such experiment indicating a high proportion (5 of 7 or 71%) of transplanted cells able to colonise the spleen contained retroviral *myb*. Similar results from other experiments will be discussed below (Table 4.3A).

4.3 ANALYSIS OF LONG TERM RECONSTITUTED RECIPIENTS

Five different experiments were conducted to observe the effects of expression of CT3Myb in the BM cells of long term reconstituted recipients.

4.3.1 Optimal culture conditions

The experiments were varied in the number of days the BM cells were maintained in culture with cytokines prior to infection by co-cultivation with Ψ2 transfectants, the total number of days in culture, and the number of days in co-culture. The different combinations tested included 1 day pre-culture followed by 3 days co-culture, 2 days of pre-culture followed by 2 days co-culture or 3 days co-culture. For each experiment, mice were reconstituted with donor cells infected with CT3*myb* or RUFneo virus, and will be referred to "myb" or "neo" recipients respectively. The co-cultivated 5FU BM cells were analysed in an *in vitro* clonogenic

assay for transformation by *myb* (see section 4.2.1) and in a CFU-S assay to assess the frequency of infection of repopulating cells (see section 4.2.3). These results are shown in Table 4.3 (A). Blood samples from long term recipients were analysed at 2 and 5 months post-transplantation in all experiments and also at 9 months in 3 experiments. The total WBC counts and the percentage of donor cells shown in Table 4.3 (B) were determined as detailed in section 2.11.2 (A). The percentages of total 'myb' mice at each time point that were found to be positive for retroviral *myb* by PCR (see section 2.11.2 (B)) are shown in Table 4.3 (B). All recipients were successfully reconstituted with donor cells and at all time points the majority of the blood cells were donor-derived. The highest infection frequency was achieved by co-cultivation for 3 days, as assessed by the greatest number of recipients positive for retroviral DNA and the highest proportion of infected CFU-S.

The percentages of surviving recipients for each of these five experiments are shown in Figure 4.6 A and B for 'myb' and 'neo' mice respectively. Overall, there were no obvious differences between the survival of recipients transplanted with BM cells infected with RUFneo compared to those with CT3myb (as was also the case in the experiments shown in Figure 4.2B and Table 4.2). Increased long term survival of recipient mice correlated with fewer days of *in vitro* culture of BM cells.

The data in Table 4.3 and Figure 4.6 suggest that the optimal *in vitro* procedure conditions of those analysed, was infection by 3 days co-culture without pre-culture. The results of individual 'myb' mice showed that the recipients that were positive for blood cells containing integrated retroviral CT3*myb* DNA, showed no difference in WBC count when compared to the control 'neo' mice, which was reflected in the average WBC counts. Results from individual mice will be discussed further below.

4.3.2 Analysis of the effects of CT3Myb in long term recipients

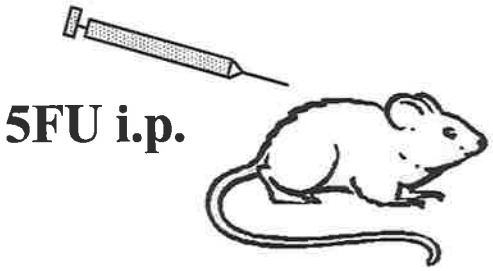
In experiment 5 discussed above, individual long term recipients were marked and their peripheral blood samples were monitored up to 9 months post-transplantation, following which their tissue samples were analysed. The analysis of peripheral blood samples from individual recipient mice at 2 months and 5 months are shown in Table 4.4(A) and (B) respectively, where the average results were shown previously in Table 4.3B. The results in Table 4.4 indicate that each mouse was successfully repopulated. Also there were no significant differences in the WBC counts of individual 'myb' mice that were positive for retroviral *myb* by PCR and 'neo' control mice or 'myb' mice negative for retroviral *myb,* as was indicated from the average results (Table 4.3). At 9 months post-transplantation peripheral blood samples were taken from each recipient to analyse the WBC counts shown in Figure 4.7(A). Subsequently, the recipient mice were killed and cells from the spleen and BM were analysed for the presence of retroviral *myb* by PCR (Figure 4.7A). The BM cells were also plated in GM-CSF, however there were no differences in the number or type of colonies formed between the mice transplanted with myb-infected cells compared to neo-infected cells (Figure 4.7A). BM from one recipient mouse (Myb2) formed a significantly higher number of colonies than the other recipients, but none of the colonies were able to give rise to long term cell lines in culture in the presence of GM-CSF. This recipient, of those reconsituted with *myb*-infected BM, did not show the presence of retroviral *myb* in peripheral blood or BM cells when analysed by PCR (see Figure 4.7A). All the other mice in this group, although positive for the presence of retroviral *myb*, were haematologically normal and showed no observable differences in their peripheral blood, spleen and BM cells, when compared to control mice.

Western Blot analysis of whole cell lysates from the spleen and BM of the 'myb' recipients shown in Figure 4.7(B), revealed that retroviral Myb protein could be detected in spleen samples from some recipients (Myb1-Myb3). Although faint, detection of CT3Myb is observed in the BM samples of the same mice. ~~it is not possible to conclude from this data if CT3Myb was expressed~~. However, it is obvious that the level of CT3Myb detected in any sample was lower than that of endogenous c-Myb. Furthermore, the level of CT3Myb detected in the spleen samples was much lower than that detected in MTHC cells maintained *in vitro*, although the level of endogenous c-Myb detected was higher. However, CT3Myb was expressed in the haemopoietic spleen cells of some 'myb' recipients, that were haematologically normal compared to control 'neo' mice.

Figure 4.1 : Outline of the bone marrow reconstitution procedure.

This procedure is described in more detail in section 2.11.1. Briefly, 5FU treated donor bone marrow cells were infected with retroviruses. The infected bone marrow cells were transplanted into lethally irradiated recipients. The recipient mice were analysed at various times post-transplantation. A fraction of the infected bone marrow cells were assessed *in vitro* by a clonogenic assay similar to that described in section 2.10.1 (C) except that the cells were cultured for 10 days in GM-CSF prior to plating.

DONOR: male (Ly5.1)

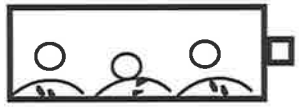


5FU i.p.

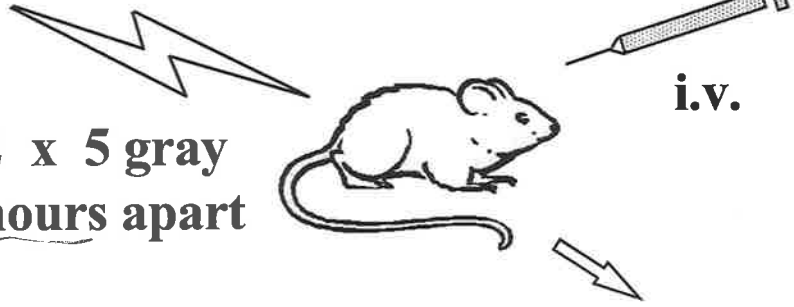
4 days

bone marrow cells (from 1 femur)

co-culture with ($1x10^6$) virus producing cells



RECIPIENT: female (Ly5.2)



2 x 5 gray 4 hours apart

i.v.

assess transformation *in vitro*

analysis of peripheral blood

Table 4.1 : Assessment of the optimal method of infection of bone marrow cells.

Donor 5FU treated bone marrow cells were infected as described in section 2.11.1C. Cells from one femur were either co-cultivated with 10^6 Ψ2 cells producing CT3Myb and RUFneo, or infected with virus-containing supernatant from the same cells. Following infection the cells were cultured for 10 days in the presence of GM-CSF, then $2x10^4$ cells/dish were plated in triplicate methylcellulose cultures. Colonies containing more than 50 cells were counted and the average +/- SEM of the results from each of 3 different experiments are shown.

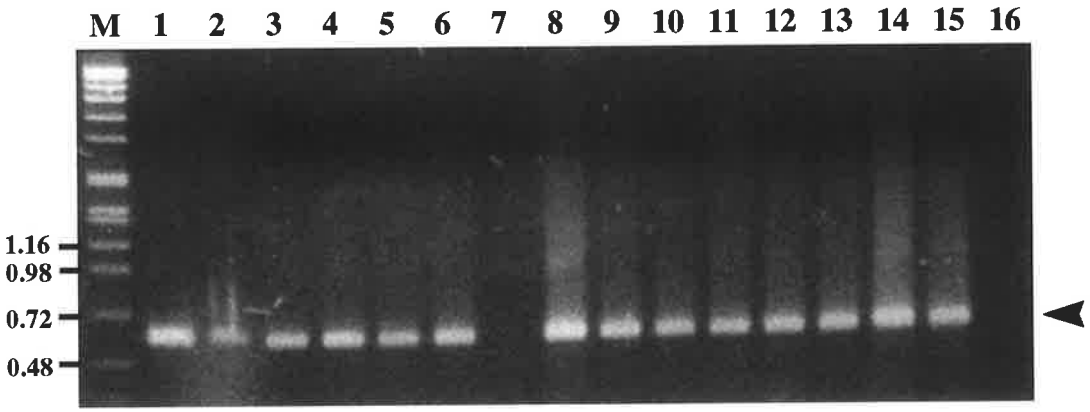
	CT3Myb	RUFneo
1 co-cultivation supernatant	73.0 ± 5.9 49.0 ± 12.2	0.0 ± 0.0 1.0 ± 0.2
2 co-cultivation supernatant	223.3 ± 18.0 71.3 ± 17.5	1.0 ± 0.7 2.0 ± 1.4
3 co-cultivation supernatant	131.5 ± 5.0 96.3 ± 8.0	4.3 ± 3.1 3.0 ± 2.1

Figure 4.2 : Detection of male donor cells in the peripheral blood of reconstituted female recipients.

A : The photograph shows an example of the PCR products observed by gel electrophoresis. The products obtained from PCR reactions using primers Zfy1 and Zfy2 on genomic DNA samples from transplanted recipients in lanes 1-14, a normal male donor in lane 15 and a normal female recipient (non-irradiated, non-transplanted) in lane 16 are shown. The sizes of the marker DNA bands are shcwn on the left and the arrow on the right points to the Y-specific product. Lanes 1-6 and 8-15 show samples that are positive for the male marker, and lanes 7 and 16 are negative.

B : The table shows the detection of the Y-specific product by PCR on genomic DNA from peripheral blood samples of recipients at various time-points post-transplantation. The '+' indicates the presence of the Y-specific product and '-' the absence. Neo1-Neo7 and Myb1-Myb7 represent 7 recipient mice that were transplantated with bone marrow cells infected with RUFneo or with CT3Myb respectively. Normal male donor and female recipient mice were used as control samples in the PCR reactions. The mice that died (of unknown causes) over the course of the experiment were Myb1, Myb5 and Neo1 that died at 2weeks, 5months and 6months post-transplantation respectively.

A



B

	2 weeks	2 months	4 months	8 months
Neo 1	+	+	+	+
Neo 2	+	+	+	+
Neo 3	+	+	+	+
Neo 4	+	+	+	+
Neo 5	+	+	+	+
Neo 6	+	+	+	+
Neo 7	+	+	-	died
Myb 1	-	died		
Myb 2	+	+	+	+
Myb 3	+	+	-	+
Myb 4	+	+	+	+
Myb 5	+	+	-	died
Myb 6	+	+	+	+
Myb 7	+	+	+	+
donor	+	+	+	+
recipient	-	-	-	-

Figure 4.3 : Examples of immunofluoresence data for expression of Ly5 markers.

The scatter plots shown in A and E represent the forward scatter versus the side scatter and expression profiles (B-D, F-L) show the relative cell number versus the relative fluorescence intensity. All the panels are representative examples of the expression of Ly5.1 (the 2 left columns) and Ly5.2 (the 2 right columns) on the peripheral blood cells of recipient mice. Panels A-H show the results of the expression analysis of an individual recipient mouse transplantated with CT3Myb infected bone marrow at 5months post-transplantation. Panels A-D show the results of peripheral blood cells incubated with Ly5.1. Panel A shows the cell scatter plot and the gated blood cell populations 1-3. Panel B, C and D show the Ly5.1 expression profile of the lymphocytes in gate 1, monocytes in gate 2 and granulocytes and gate 3 respectively. Panels E-H show the results of peripheral blood cells incubated with Ly5.2. Panel E shows the cell scatter plot and the gated populations 1-3. Panel F, G and H show the Ly5.2 expression profile of the lymphocytes in gate 1, monocytes in gate 2 and granulocytes and gate 3 respectively.

Panels I and J show the Ly5.1 expression profiles of gated lymphocytes from normal donor and normal recipient control mice respectively. Panels K and L show the Ly5.2 expression profiles of gated lymphocytes from the same normal donor (Ly5.1+) and normal recipient (Ly5.2+) control mice. The profile in panel J was used as a negative control to set the marker line shown past the peak for analysis of the percentage of cells positive for Ly5.1 expression in all the other samples. Similarly, the profile in panel K was used as a negative control to set the marker line shown past the peak for analysis of the percentage of cells positive for Ly5.2 expression in the other samples.

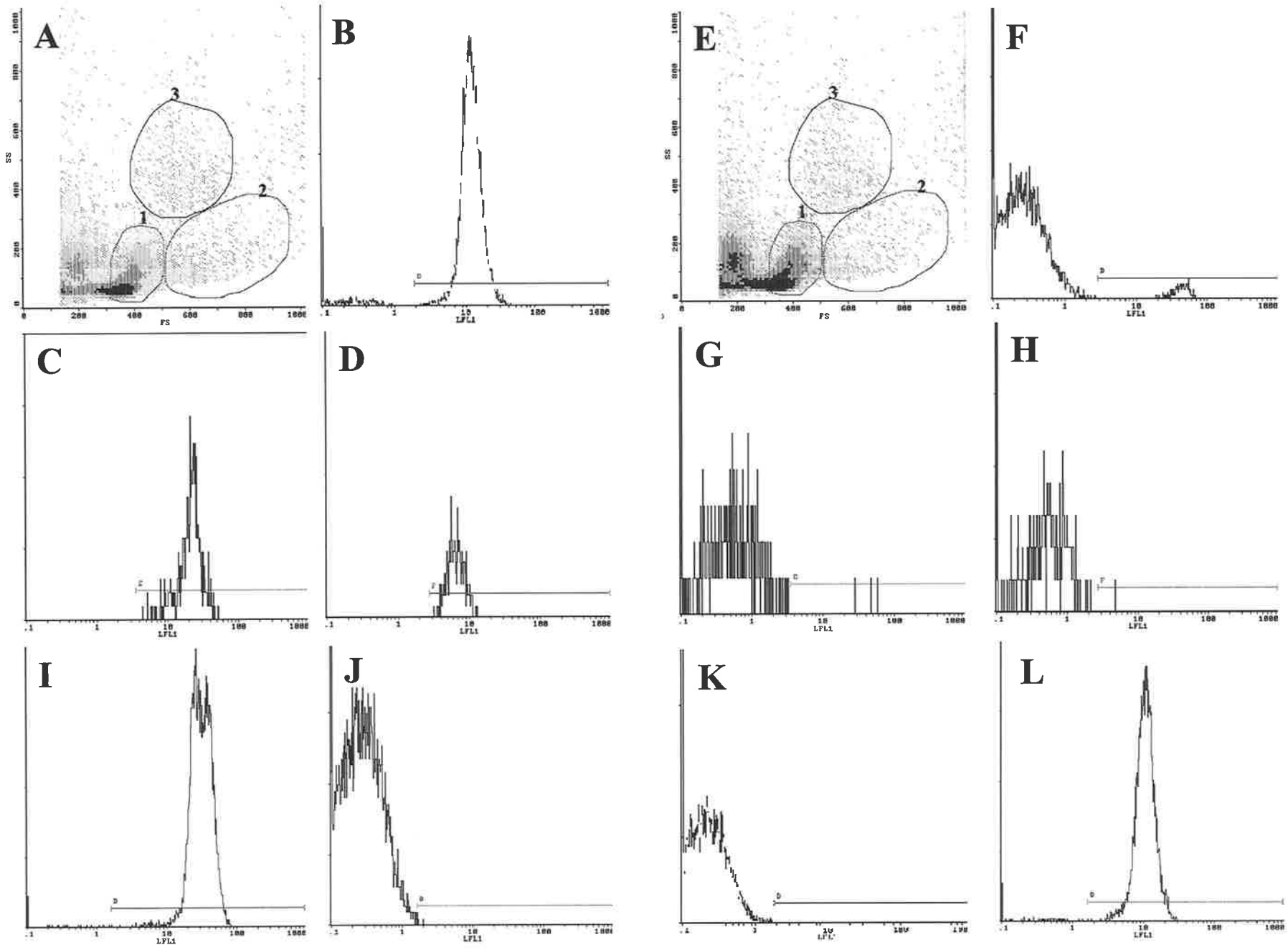

A
B
C
D
E
F
G
H
I
J
K
L
1
2
3
SS
FS
LFL1

Table 4.2 : Detection of the Ly5.1 and Ly5.2 markers on the peripheral blood cells of recipient mice.

The average percentage of peripheral blood cells (lymphocytes, monocytes and genulocytes) positive for expression of the Ly5.1 or Ly5.2 at various times post-transplantation are shown for all reconstituted recipients in one experiment. Neo1-Neo5 and Myb1-Myb5 represent five recipient mice that were transplantated with bone marrow cells infected with RUFneo or CT3Myb respectively. A non-irradiated, non-transplanted (Ly5.2+) recipient mouse was used as a control. 'Neo 4' and 'Myb 5' mice died over the course of the experiment (of unknown causes) at 4 and 6 months post-transplantation respectively. ND represents not done.

	% cells Ly 5.1+			% cells Ly 5.2+		
	1 month	5 months	8 months	1 month	5 months	8 months
recipient	1.5	2.7	0.5	99.4	98.9	80.8
Neo 1	93.2	99.5	89.7	7.6	8.5	0.9
Neo 2	82.6	95.3	98	7.6	10.2	2.1
Neo 3	90.8	98.5	95.7	11.9	1.8	3
Neo 4	79.6	61.1	died	6.3	8	died
Neo 5	90.3	97.8	ND	5.5	2.3	ND
Myb 1	93.7	96.9	97.6	6.6	2.3	3.1
Myb 2	91.5	95.9	95.3	13.8	5.1	6
Myb 3	91.7	97.5	96.6	16	2.6	3.1
Myb 4	92.9	97.7	95.6	9.4	2.1	1.8
Myb 5	93.2	died	died	6.1	died	died

Figure 4.4 : Detection of retroviral DNA in the peripheral blood of long term reconstituted recipients.

The photographs shown are examples of PCR analysis for retroviral DNA in genomic DNA samples from peripheral blood of recipients.

A: The products obtained from PCR reactions using primers MybA and MybS on genomic DNA samples from recipients transplanted with CT3Myb infected bone marrow and RUFneo infected bone marrow are shown in lanes 2-6 and lanes 7-10 respectively. Genomic DNA from a normal recipient was used as a negative control for retroviral *myb* as shown in lane 1, DNA from a MTHC cell line was used as a positive control for retroviral *myb* in lane 11 and a reaction with no added DNA as a PCR control in lane 12. The sizes of the marker DNA bands are shown on the left and the arrows on the right point to the endogenous (E) and retroviral (R) products. Lanes 2, 5, 6 and 11 show samples that are positive for the retroviral *myb* product, and lanes 1, 3, 4, 7-10 and 12 negative. All lanes from 1-10 are positive for the endogenous *myb* product, but lane 11 shows a different size faint band of ~200bp, which could reflect a polymorphism in the intron since all blood samples (Lanes1-10) were derived from C57 strain mice whereas the MTHC sample (Lane 11) was derived from a CBA strain mouse.

B: Southern Blot of the PCR products with a *myb* specific probe. Lane1 shows the no DNA PCR control, lanes 2-6 show the PCR products from the same samples shown in lanes 2-6 of Panel A, and lane 7 shows the positive control shown in lane 11 of Panel A. **All lanes shown were from the same blot.**

C: The products obtained from PCR reactions using primers Neo1 and Neo2 on gemonic DNA from peripheral blood samples of recipients transplanted with RUFneo infected bone marrow. The sizes of the marker DNA bands are shown on the left and the arrow on the right points to the neo specific product. Lanes 2-5 show the neo PCR products amplified from the same samples that were shown in lanes 7-10 of Panel A. A reaction with no added DNA as a PCR control is shown in lane 1.

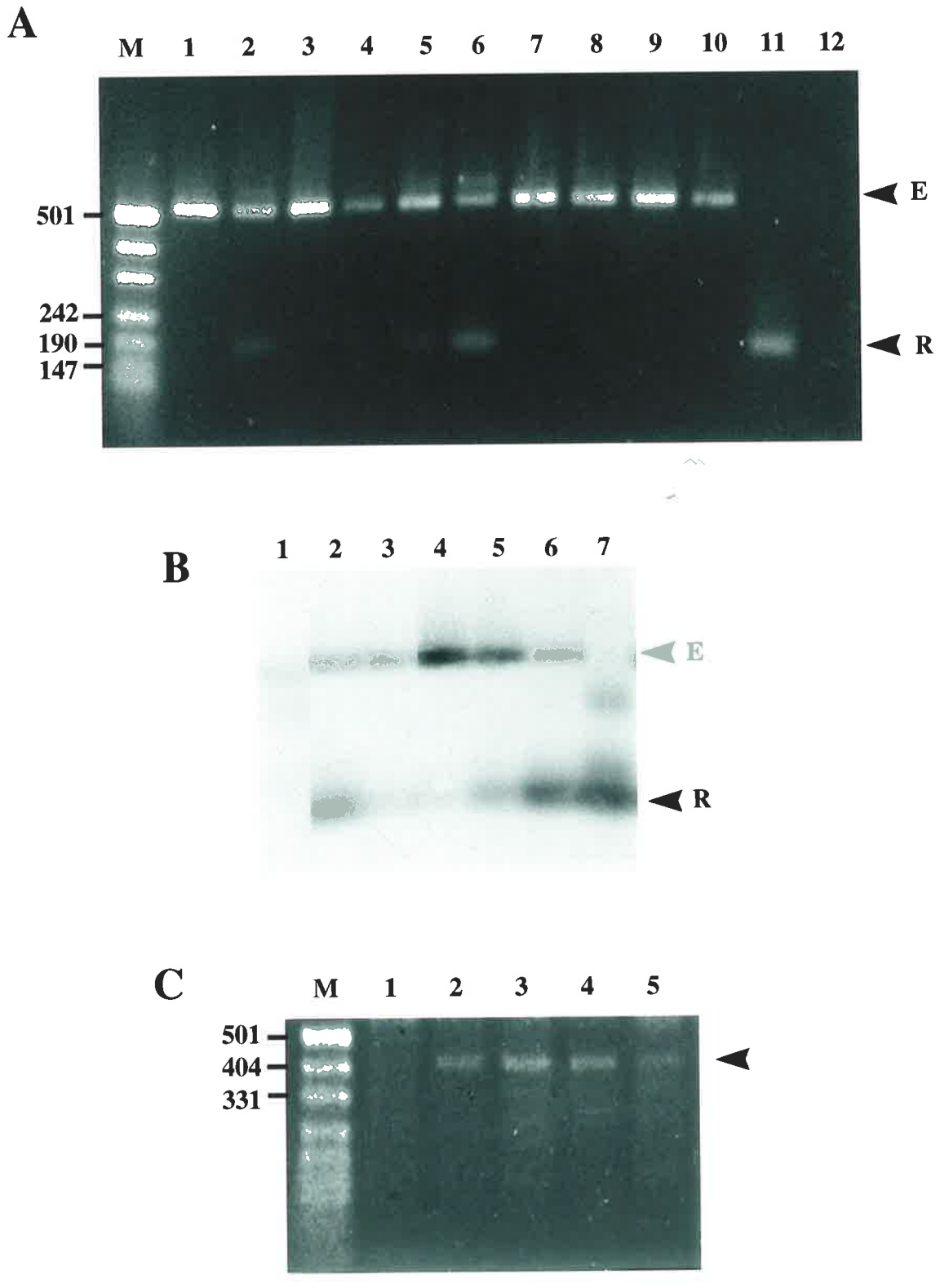

A
M 1 2 3 4 5 6 7 8 9 10 11 12
501
242
190
147
E
R
B
1 2 3 4 5 6 7
E
R
C
M 1 2 3 4 5
501
404
331

Figure 4.5: Detection of retroviral *myb* in individual CFU-S.

Genomic DNA extracted from cell suspensions obtained from individual day 12 CFU-S were analysed by PCR using the MybA and MybS primers. The photograph shows an example of a Southern Blot of the PCR products obtained from 7 spleen colonies (lanes 2-8) from a recipient transplanted with CT3Myb-infected BM . Lane 1 shows the result of a reaction with no genomic DNA as a PCR control and lane 9 shows a peripheral blood sample (from a long term recipient that was previously assessed as being positive) used as a positive control. Lanes 2,3,5,7-9 are positive for the retroviral Myb product (labelled 'R' on the right) and lanes 2-9 are positive for the endogenous Myb product (labelled 'E').

The endogenous Myb band was used as an internal control for the level of protein in each sample. The sizes of two marker DNA bands are shown on the left.

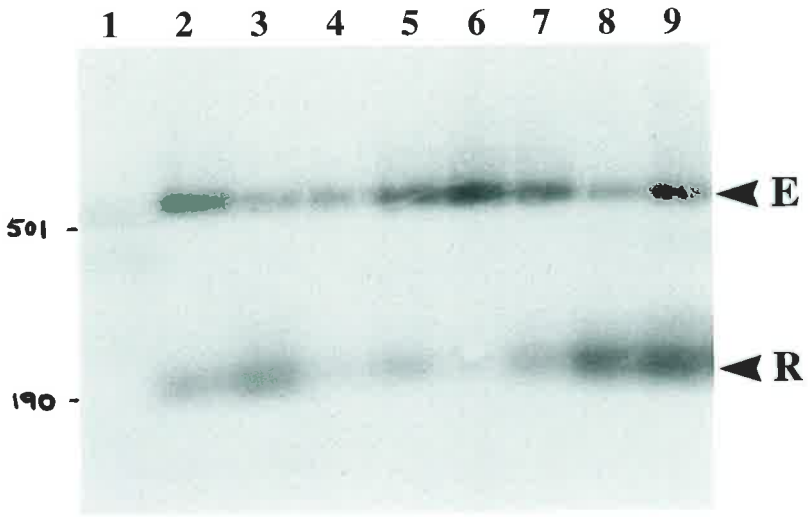

1 2 3 4 5 6 7 8 9
501
190
E
R

Table 4.3 : Analysis of infected donor bone marrow cells and long term recipients at various times post-transplantation.

The data shown are derived from 5 different reconstitution experiments in which the infection procedures differed with respect to the days of pre-culture with cytokines (XdaysP) and the number of days in co-culture with Ψ2 cells and cytokines (XdaysC), as defined in Table A. In each eperiment 5 mice were reconstituted with RUFneo infected bone marrow and 5 with CT3myb infected bone marrow

A: Assessment of infection of bone marrow cells by CT3*myb* retrovirus. Following culture in GM-CSF for 10 days post-infection, the pooled bone marrow cells were plated in methylcellulose supplemented with GM-CSF, and the average number of colonies formed by 20,000 cells after one week are indicated as the 'CFU(Neo)' and 'CFU(Myb)' for RUFneo or CT3Myb infected populations respectively. The cells were also assessed by a day 12 CFU-S assay in recipient mice. The percentages of spleen colonies positive for retroviral myb, as analysed by PCR on samples from a recipient transplanted with CT3Myb-infected BM, are shown as '% CFU-S +'.

B: Analysis of peripheral blood samples from long term recipients. Results are shown as the average +/- the SEM for all mice in each experimental group. The average percentage of peripheral blood white blood cells positive for expression of Ly5.1 and Ly5.2 are shown as '%Ly5.1 +' and '%Ly5.2 +'. The average total white blood cell counts for mice transplanted with bone marrow infected with RUFneo or CT3myb, are shown as WBC (Neo) and WBC (Myb) respectively. The percentage of mice transplanted with myb infected bone marrow that were positive for retroviral myb as determined by PCR, are shown as '% PCR +'.

A

Experiment	*In vitro* CFU (Neo)	*In vitro* CFU (Myb)	% CFU-S Myb +
1 (1dayP, 3daysC)	1.3 +/- 1.1	38.7 +/- 3.6	ND
2 (2daysP, 2daysC)	6.2 +/- 1.1	109.7 +/- 13.1	40 (4 of 10)
3 (0daysP, 3daysC)	4.3 +/- 0.8	129.0 +/- 4.6	55 (5 of 9)
4 (0daysP, 3daysC)	10.0 +/- 1.9	80.0 +/- 9.7	67 (4 of 6)
5 (0daysP, 3daysC)	10.6 +/- 0.4	74.3 +/- 4.7	ND

B

Experiment : Time	% Ly 5.1 +	% Ly 5.2 +	WBC (Neo)	WBC (Myb)	% PCR +
Exp. 1 : 2 months	ND	ND	8.0 +/- 0.7	4.0 +/- 1.9	66
: 5 months	ND	ND	6.3 +/- 2.3	4.7 +/- 1.8	66
Exp. 2 : 2 months	89.1 +/- 3.3	8.8 +/- 1.3	ND	ND	0
: 5 months	93.3 +/- 4.2	4.0 +/- 1.1	5.1 +/- 1.1	6.3 +/- 0.9	0
: 9 months	95.5 +/- 1.1	3.3 +/- 0.6	7.7 +/- 0.3	6.3 +/- 1.0	0
Exp. 3 : 2 months	95.5 +/- 1.5	2.7 +/- 0.5	9.5 +/- 1.8	7.1 +/- 0.6	60
: 5 months	91.7 +/- 2.9	3.3 +/- 1.3	7.0 +/- 1.1	5.6 +/- 1.1	75
Exp. 4 : 2 months	90.9 +/- 1.2	11.5 +/- 1.2	3.4 +/- 0.5	3.8 +/- 0.7	66
: 5 months	95.3 +/- 1.2	3.5 +/- 0.4	11.3 +/- 1.5	11.5 +/- 0.7	50
: 9 months	92.7 +/-1.1	5.2 +/- 1.9	6.0 +/- 2.2	7.5 +/- 0.8	50
Exp. 5 : 2 months	84.5 +/- 2.5	12.0 +/- 0.7	5.4 +/- 0.3	6.4 +/- 0.7	60
: 5 months	92.8 +/- 2.7	6.8 +/- 1.8	6.6 +/- 0.6	7.8 +/- 0.5	80
: 9 months	ND	ND	7.4 +/- 0.6	8.0 +/- 1.3	80

Figure 4.6: The survival of long term reconstituted recipients over 10 months post-transplantation.

The graphs in Panel A and B show the percentage of surviving 'myb' and 'neo' long-term recipients respectively from five different experiments. Experiments 1-5 shown correspond to those shown in Table 4.3. Experiments 3 and 5 were terminated at 7 and 9 months post-transplantation respectively for analysis of tissues samples.

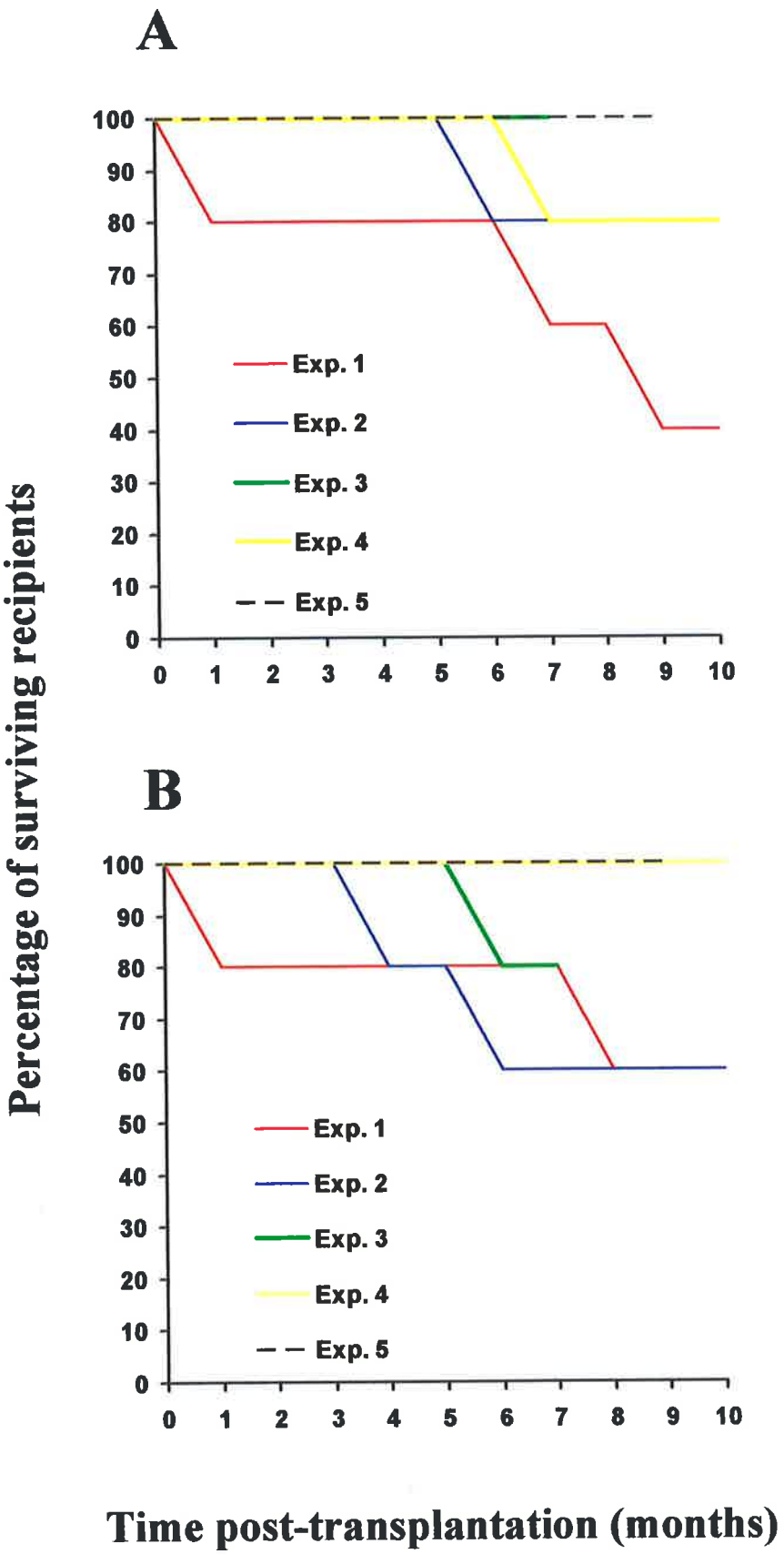

A
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10
Exp. 1
Exp. 2
Exp. 3
Exp. 4
Exp. 5
B
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10
Exp. 1
Exp. 2
Exp. 3
Exp. 4
Exp. 5
Percentage of surviving recipients
Time post-transplantation (months)

Table 4.4: Analysis of 10 individually marked long term recipients.

Analysis of the individual recipients transplanted with BM infected with RUFneo (Neo1-Neo5) or CT3Myb (Myb1-Myb5) in a single experiment, previously discussed as Experiment 5 (Table 4.3, Figure 4.6)

Tables A and B show the analysis of each long term recipient at 2 and 5 months post-transplantation respectively. The tables show the analysis of peripheral blood samples from reconstituted recipient mice Neo1-Neo5 and Myb1-Myb5 and from a normal recipient and a normal donor mouse as controls. The total white blood cell count 'WBC' and the percentage of blood cells positive for Ly5.1 (%Ly 5.1) or Ly5.2 (%Ly 5.2) are shown. The detection of retroviral *neo* (Neo PCR +) or *myb* (Myb PCR +) by PCR analysis on genomic DNA samples from the recipients are indicated as a '+', the absence of specific products are indicated as '-' and (nd) indicates that the PCR reaction was not done.

A	WBC	% Ly 5.1	% Ly 5.2	Neo PCR +	Myb PCR +
Neo 1	6.0	89.7	9.8	+	-
Neo 2	5.0	85.7	14.9	-	-
Neo 3	6.0	88.2	10.5	+	-
Neo 4	5.2	90.2	9.3	+	-
Neo 5	4.7	80.5	15.6	-	-
Myb 1	6.6	69.7	10.5	(nd)	-
Myb 2	4.0	73.9	12.5	(nd)	-
Myb 3	6.7	87.4	13.1	(nd)	+
Myb 4	7.1	89.6	12.6	(nd)	+
Myb 5	7.4	90.3	10.7	(nd)	+
Recipient	6.0	2.5	99.1	-	-
Donor	10.2	99.1	0.6	(nd)	(nd)

B	WBC	% Ly 5.1	% Ly 5.2	Neo PCR +	Myb PCR +
Neo 1	7.0	96.9	4.7	+	-
Neo 2	4.5	96.3	6.7	-	-
Neo 3	6.8	97.9	4.2	+	-
Neo 4	7.9	98.1	4.1	+	-
Neo 5	6.9	96.8	4.3	-	-
Myb 1	8.3	90.8	4.4	(nd)	+
Myb 2	9.1	82.1	7.2	(nd)	-
Myb 3	6.9	74.5	21.4	(nd)	+
Myb 4	7.0	97.3	5.3	(nd)	+
Myb 5	7.9	97.4	5.3	(nd)	+
Recipient	9.3	1.1	97.4	-	-
Donor	10.0	95.1	0.2	(nd)	(nd)

Figure 4.7: Analysis of peripheral blood and tissue samples obtained from long term recipients of a single reconstitution experiment at 9 months post transplantation.
Each recipient shown here corresponds to those shown in Table 4.4 and represent the individual recipients from Experiment 5 (Table 4.3, Figure 4.6).

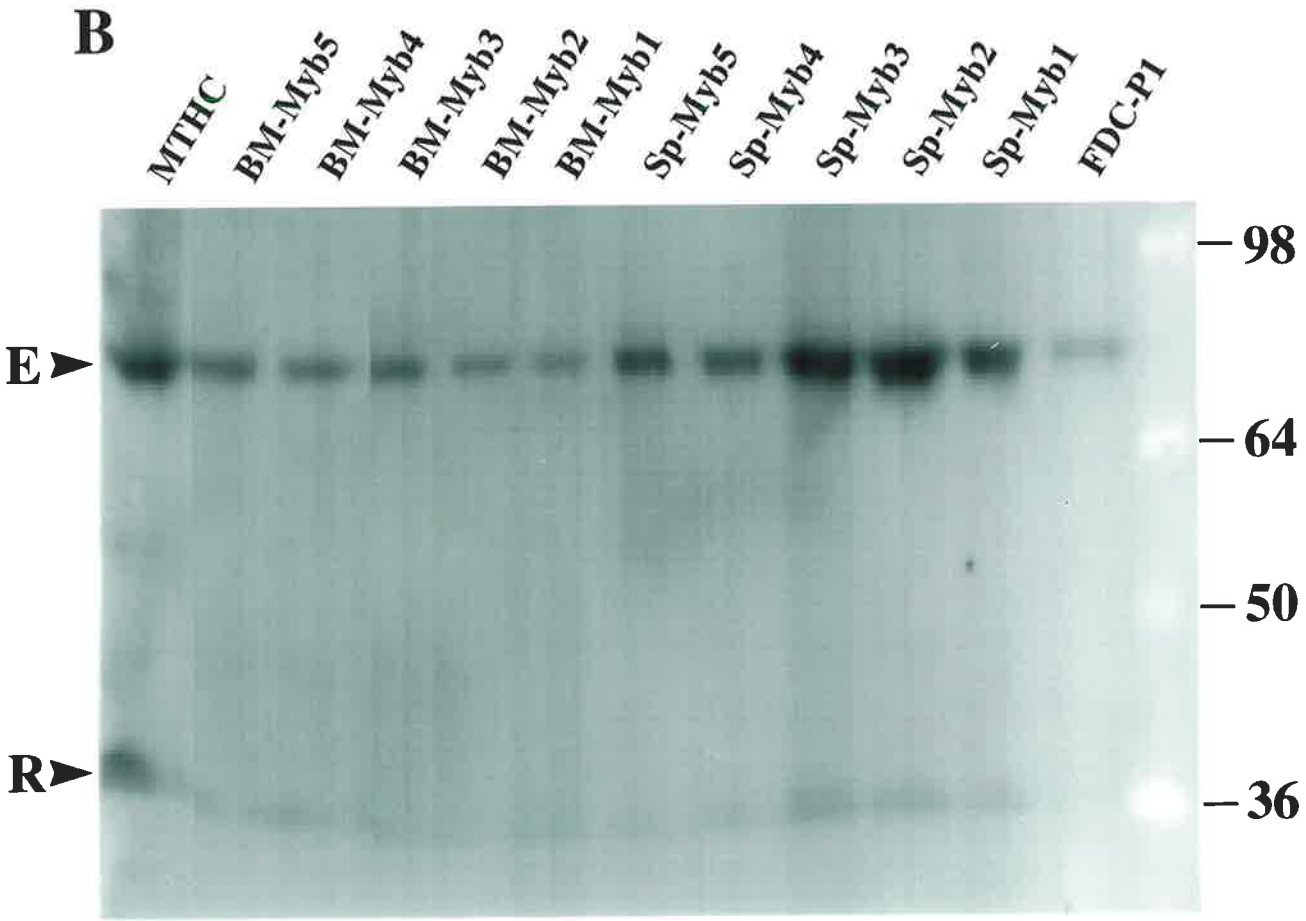
A: The table shows the total white blood cell count 'WBC' for each recipient as well as for control mice (similar to Table 4.4). The bone marrow cells from each recipient were plated at 10,000 cells per dish in GM-CSF and after 1 week, the average number of colonies (>50 cells) per dish +/- the SEM for triplicates are shown under 'B.M. colonies'. Genomic DNA samples from the peripheral blood, spleen and bone marrow of each recipient were analysed for retroviral *myb* by PCR. The results are shown under the headings of 'Blood', 'Spleen' and 'Marrow' and '+' represents the detection of a retroviral product, '-' the absence of a retroviral PCR product (where an endogenous product was detected) and 'nd' represents not done.

B: Western Blot Analysis of tissue samples for Myb proteins. Whole cell lysates from the spleen and bone marrow from recipients transplanted with CT3myb-infected bone marrow were analysed for Myb protein expression with biotin-mAb 5.1 and streptavidin-AP (see section 2.7.1). The results shown were obtained by detection of fluorescence using a Fluoro-Imager. The lanes are labelled as 'BM' and 'Sp' representing either bone marrow or spleen samples obtained from recipients Myb1-Myb5. MTHC and FDC-P1 cells were used as a positive control and negative control for retroviral Myb protein. 'E' indicates the size of the endogenous c-Myb protein and 'R' indicates the size of the retroviral CT3Myb protein.

A

	WBC	B.M. colonies	Blood	Spleen	Marrow
Neo 1	7.4	14.5 +/- 2.1	-	-	-
Neo 2	6.2	20.0 +/- 2.8	-	-	-
Neo 3	8.8	28.0 +/- 2.8	-	-	-
Neo 4	(nd)	29.0 +/- 5.7	-	-	-
Neo 5	7.2	20.5 +/- 3.5	-	-	-
Myb 1	5.9	14.5 +/- 3.5	+	+	+
Myb 2	10.4	55.5 +/- 5.7	-	+	-
Myb 3	9.3	17.0 +/- 7.1	+	+	+
Myb 4	(nd)	22.0 +/- 1.0	+	+	+
Myb 5	6.5	28.0 +/- 2.8	+	+	+
Recipient	4.8	(nd)	-	-	-
Donor	9.1	(nd)	(nd)	(nd)	(nd)

B



4.4 DISCUSSION

The transplantation of lethally irradiated recipients with donor BM has been utilised as a procedure to repopulate the haemopoietic system of mice with genetically manipulated cells in many studies (reviewed in Bonham and Symonds, 1992). Our experiments proved we were able to duplicate this procedure successfully by detecting blood cells of donor origin in recipients up to 10 months post-transplantation.

One of the aims of this study was to examine the outcome of reconstituting the haemopoietic system of recipient mice with BM cells continuously expressing activated Myb. In most of the experiments the presence of retroviral *myb* was detected in the peripheral blood of recipient mice transplanted with myb-infected BM. However no differences in the peripheral blood WBC count or cellularity in comparison to control mice were observed. In experiments where the haemopoietic tissues of the mice were analysed, there were no obvious abnormalities in the spleen or BM cells of recipients transplanted with "*myb* infected" cells compared to control recipients with "*neo* infected" cells. Analysis of the expression of retroviral Myb protein revealed that although the level of CT3Myb protein detected in the haemopoietic spleen cells of some recipient mice was lower than that of endogenous c-Myb, CT3Myb was expressed.

The absence of any effect due to expression of an oncogene in reconstituted mice is not unique to this study. Transplantation of BM expressing the *scl* gene (a transcripition factor) into long term recipient mice did not result in leukaemia or any alteration in the WBC count even though the retroviral gene was detected in the peripheral blood of recipients for at least 9 months post-transplantation (Elwood and Begley, 1995).

The inability of continuous expression of CT3Myb *in vivo* to lead to some kind of haemopoietic abnormality could be explained in several ways. One reason could be that the level of expression of CT3Myb was insufficient to cause any effect. However, it is not known whether the CT3Myb protein detected in the recipient mice tissue samples was obtained from a few cells expressing high levels or many cells expressing low levels. This posed a problem, since more detailed analysis such as single cell RTPCR would determine if all cells were expressing CT3Myb but would not assess the level of protein expressed. Other immunocytochemical analysis of the level of protein expression would not distinguish between endogenous c-Myb and CT3Myb. Hence, if the level of protein was a critical factor in these experiments, it is difficult to predict whether an increase in the expression levels of CT3Myb or more target cells expressing Myb would be required to observe any effects.

Another possible reason that no effects were observed due to CT3Myb, is that insufficient numbers of recipients were examined to detect oncogenic potential. However, in a similar experiment using the same procedure and similar conditions (including the same retroviral expression vector) as those in this study, recipients reconstituted with BM cells infected with activated forms of the GM-CSF receptor β chain showed haematological differences compared to control mice. Also, all recipients (6) transplanted with BM infected with the activated mutant showed haematological differences in the erythroid and myeloid lineages after 1 month and 3 months post-transplantation respectively, when compared to controls (M. M^{c}Cormack and T. Gonda - unpublished data). Therefore it seems unlikely that the absence of any detected haematological disorder in 'myb' mice was due insufficient numbers of recipients examined in this study.

A more likely explanation, is that continuous expression of Myb in haemopoietic cells is not sufficient for transformation and subsequent mutations in other oncogenes may be necessary. Avian myeloid cells transformed by E26 are dependent on exogenous factor for growth (Beug *et al.,* 1982). Studies of growth *in vitro* of cell lines derived from ABML/MML tumours (see section 1.3.2 and Figure 1.2) indicated that acquisition of CSF-independence was probably a secondary step in transformation by v-Myb (Leslie and Schrader, 1989). Murine cell line NFS-60 (see Figure 1.2)(Weinstein *et al.,* 1986) and primary murine cells transformed by recombinant *myb* retroviruses (Gonda *et al.,* 1989a) are dependent on growth factors for survival and proliferation. In the case of avian retrovirus AMV (see section 1.2.1 and Figure 1.2), it was shown that activation of a growth factor gene was necessary for leukaemogenesis that resulted from infection, even where virus spread occured *in vivo* (Metz *et al.,* 1991). In the murine system, experiments involving infection with Mo-MLV, resulted in the expression of Gag-Myb fusion protein in the spleen and/or BM of some infected mice that did not show any evidence of leukaemia, and in other mice Gag-Myb was detected prior to the development of acute promonocytic leukaemia. Hence activation of c-*myb* by pro-viral insertion was found to be an early event in MML (Nason-Burchenal and Wolff, 1993). Furthermore, in transgenic mice expressing v-Myb^{AMV} specifically in T cells, only a small proportion of older mice developed T cell malignancies (Badiani *et al.,* 1997). All these *in vitro* and *in vivo* studies suggest that activation of Myb is probably an early event in the development of leukaemia and that factor-independence is a critical subsequent step for 'complete' transformation. Therefore, it is likely that in experiments discussed in this chapter, the animals did not aquire additional spontaneous oncogenic mutations besides the activation of Myb and therefore showed no signs of any haemopoietic disorder. To

address this, increased numbers of long term recipients assessed over a longer time-course is necessary. Additionally, exposure to mutagens following reconstitution and introduction of other oncogenes (providing factor-independence) in co-operation with Myb, would also be of interest.

In the case of most cancers, the establishment of a disease state is caused by at least two stages, each of which involve activation of different proto-oncogenes (see section 1.6). Hence, it is possible that oncogene co-operativity may be necessary to observe any effects due to activation of Myb. Ideal candidates for co-operation are those which provide factor-independence, for example expression of autocrine GM-CSF (Gonda *et al.,* 1989a) has been shown to confer tumourigenic potential on MTHC. The Myb-Ets fusion protein is the known transforming factor of E26 and fusion of the two have been shown to be necessary for leukomogenicity of E26 (Metz and Graf, 1991b). In addition, the Ras signalling protein has been shown to co-operate with Myb in transformation of hampster embryonic cells (Merzak *et al.,* 1992). The co-operation between Myb and activated c-Kit receptor has been investigated and will be discussed in the following chapters.

Chapter 5. EXPRESSION OF ACTIVATED KIT IN PRIMARY CELLS

5.1 INTRODUCTION

A constitutively activated form of c-Kit was first identified in the human mast cell line HMC-1 (Furitsu *et al.,* 1993) as discussed previously in section 1.5.2. An amino acid alteration in the tyrosine kinase domain caused by a point mutation was also detected in the corresponding residues of Kit expressed in murine and rat mast cell lines, (Tsujimura *et al.,* 1994, Tsujimura *et al.,* 1995) suggesting that this residue was critical for Kit activity. This mutant form of Kit was able to confer factor-independence in the murine myeloid cell line FDC-P1 and mast cell line IC-2, as well as inducing differentiation of IC-2 cells (Kitayama *et al.,* 1995, Hashimoto *et al.,* 1996). Our ultimate aim was to determine the co-operative effects of activated Myb and activated Kit in haemopoietic cells. In order to fully understand of the co-operative effects of the two oncogenes, it was necessary to first examine each individually. The effects of over-expression of activated Myb in primary FLC are well known (as discussed in the previous chapters), but the effects of activated Kit in these cells have not been previously documented.

A recent study introducing normal and activated forms of c-Kit into the BM of mice showed that expression of activated murine KIT^{V814} could result in acute lymphoblastic leukemias. *In vitro* colony assays showed that introduction of KIT^{V814} into BM cells gave rise to factor independent granulocyte / macrophage, mast and mixed colony forming cells (Kitayama *et al.,* 1996). However, KIT^{V814} caused a decrease in GM colonies formed in IL-3 or SCF compared to control cells (Kitayama *et al.,* 1996). Furthermore, the frequency and monoclonality of lymphoid leukemias arising in these mice indicated that KIT^{V814} alone did not transform the cells (Kitayama *et al.,* 1996). The nature of the malignancy observed in these animals may

reflect the predisposition of different lineages to subsequent activation of other oncogenes. Constitutively activated mutant Kit^{Y814} has also been shown to function differently to c-Kit activated by SCF, displaying different substrate specificities and the ability to result in degradation of HCP ~~phosphatase~~ (Piao *et al.,* 1996). Therefore, in the work described in this chapter, the effects of mutant Kit in FLC were compared to normal FLC in the absence of factor, as well as WT c-Kit in the presence of SCF. The co-operative effects of Kit and Myb in FLC was also addressed.

5.2 GENERATION OF KIT CONSTRUCTS AND ANALYSIS OF EXPRESSION

5.2.1 Generation of Kit expression constructs

Some FLC express endogenous murine Kit, hence human Kit was used in these experiments since antibodies to human Kit (that do not recognize murine Kit) would enable detection of cells expressing the introduced Kit protein, and growth in ligand huSCF (that is inactive on murine Kit - Lev *et al.,* 1993) would allow selective activation of introduced huKit but not endogenous muKit.

A cDNA clone of human c-*kit* in pBluescript (encoding the GNNK+ isoform; Crosier *et al.,* 1993) was obtained from Dr. D. Williams, Immunex, Seattle, USA. The c-kit cDNA was excised as an Asp718 - Not1 fragment from the vector by restriction enzyme digestion, subsequently blunt ended by end-filling using Klenow polymerase and subcloned into the Hpa1 site in the polylinker of pRUFneo (Rayner and Gonda 1994; see Figure 2.1) to give pRUFneo(WTkit). To confirm the orientation of the Kit cDNA, plasmid DNA clones were digested with HindIII. The c-kit cDNA of ~3Kb contains a single HindIII site at ~ 400bp from the 5' end and pRUFneo of 5.66Kb contains a single HindIII site 3' of the Hpa1 site in the polylinker (see Figure 2.1).

The sizes of the fragments obtained after digestion were used to select a clone containing the c-kit cDNA in the correct orientation for expression from the 5'LTR of pRUFneo.

A point mutation of A to T at bp 2468 of human *kit* resulting in an amino acid change from Asp to Val at residue 816 in the tyrosine kinase domain of Kit was reported to be highly activating (Kitayama *et al.*, 1996; Hashimoto *et al.*, 1996). The V816 mutation was constructed by replacing a fragment of normal *kit* cDNA with the mutant sequence derived by RT PCR. The HMC-1 cell line contained one allele with this mutation and one normal allele. Total RNA was isolated from HMC-1 cells and used as template for 1st strand cDNA synthesis. RT PCR using oligos 1002 and SRC-05 (see table 2.1, 2.2) as primers was used to obtain a 715bp product (nucleic acid residues 2167-2882 of human *kit*). The PCR product was digested with restriction endonuclease EspI (CelII) to provide a 360bp fragment (residues 2330-2690) which was separated by gel electrophoresis and purified. The pRUFneo(WTkit) plasmid was also digested with EspI, dephosphorylated, separated by gel electrophosesis and purified. The PCR fragment was directionally cloned into the EspI sites of pRUFneo(WTkit). The mutation of A to T at 2468 bp results in the loss of a BsmAI site. Hence DNA from bacterial clones was extracted and screened for the mutation by PCR using oligos SRC-09 and SRC-10 (see Table 2.1, 2.2) and restriction digestion of the product with BsmAI. The construct, pRUFneo(V816kit) was sequenced across the cloned PCR product to confirm the mutation. Figure 5.1 shows the sequence from residues 2457 to 2489 containing the A to T change at bp 2468 in the clone selected.

5.2.2 Generation of virus producing cells and assessment of viral titre

All plasmids used for transfection were expanded and purified as described in

section 2.4.10 (D). Ψ2 cells were transfected with either the pRUFneo(WTkit) or pRUFneo(V816kit) expression constructs and selected with G418 (see section 2.8.2). Expression of human Kit on transfected cell pools was detected by immunofluorescence using mAb IDC3. The transfectants expressing the highest levels of Kit were isolated by fluorescence - activated cell sorting. Of the total population, 1.8% of V816kit and 10.5% of WTkit transfected cells were positive for surface expression when compared to non-transfected cells. The top 5% and 10% of cells respectively were selected and expanded. Fluoresence histograms showing human Kit surface expression by the sorted pools are shown in Figure 5.2A. The sorted cells Ψ2 cells were used as a source of retrovirus for all infections of target cells.

The viral titre of supernatant from the sorted Ψ2 cells was determined by infection of NIH-3T3 cells as described in section 2.8.3 (A) and was calculated to be $2x10^5$ cfu/ml for the V816*kit* viral producers and $1.5x10^6$ cfu/ml for the WT*kit* viral producers.

5.2.3 Analysis of Kit expression and activity in FDC-P1 cells

The WT*kit* and V816*kit* virus producers were used to infect FDC-P1 cells as outlined in section 2.8.3 (B) by co-cultivation of $2x10^5$ FDC-P1 cells and $5x10^5$ virus producers per dish in duplicate. The infected FDC-P1 cells were selected in 1mg/ml of G418 until uninfected cells were non viable. Figure 5.2B shows the Kit surface expression profiles of $G418^R$ infectants. The WTKit cells were all positive for expression as indicated by the peak shift. However V816Kit cells were heterogeneous for huKit expression with the majority detected as negative for expression with only a small proportion of cells positive.

To determine if V816Kit was able to confer factor-independence in FDC-P1 cells, as observed in a previous study (Kitayama *et al.*, 1995), the $G418^R$ populations

were plated in methylcellulose in the presence of GM-CSF and in the absence of factor. The results shown in Figure 5.3 indicated that only a small fraction of the infected cells were factor-independent. At first this seemed to correlate with the small proportion of cells that were positive for surface expression of Kit. However, when V816Kit infected cells (the $G418^R$ population) which had been selected for growth in the absence of factor were analysed by immunofluoresence and flow cytometry, expression of human Kit on the cell surface was still below the limit of detection (Figure 5.2C). A low level of surface expression has also been observed in other studies with constitutively activated Kit and Fms receptors; this may be due to continuous turnover of the activated receptor (Glover at al 1995; Kitayama *et al.,* 1995; Moriyama *et al.,* 1996). Furthermore, the constitutively active receptor may be functional without reaching the cell surface. Likewise, WTKit infected cells selected for growth in huSCF also showed a decrease in the level of surface expression relative to the unselected population, which may be due to receptor turnover induced by SCF (Figure 5.2C).

5.3 OVER-EXPRESSION OF V816KIT AND WTKIT IN PRIMARY FLC

5.3.1 Clonogenic potential of Kit infected cells

In order to observe the effects of activated Kit, day 14 FLC were co-cultivated over 2 days with the Ψ2 transfectants (see above) producing virus containing V816kit, WTkit, CT3Myb or RUFneo and were plated in methylcellulose in the presence of muGM-CSF or the absence of factor as described in section 2.9.2. As mentioned earlier, one of the aims of this study was to determine the the co-operative effects of oncogenes Myb and Kit, and it was important to firstly asssess each individually. In contrast to the experiments described in Chapter 3 (where the effects of CT3Myb are

known), in these experiments FLC were not cultured in GM-CSF, but plated immediately following infection. Hence, the effects of CT3Myb alone was assessed in these experiments. The numbers of colonies formed after 14 days in 3 separate experiments are shown in Figure 5.4A. In the presence of GM-CSF, CT3Myb infected cells were able to form a high number of colonies, consistent with previous experiments (see chapter 3). However, WTKit and V816Kit expressing cells showed a decrease in colonies formed with GM-CSF even compared to the control (vector alone) infected cells. In the absence of factor none of the cells infected were able to form colonies, however FLC infected with V816Kit virus were able to form a few clusters of cells. A typical cluster is shown in Figure 5.4C in comparison to the usual type of colony observed in GM-CSF (Figure 5.4B). The presence of single live cells as well as in clusters in the dishes, indicated that although V816Kit infected cells were unable to form colonies in the absence of factor, some cells were able to survive. The cells in the clusters were of varying size, and appeared more differentiated than those in colonies formed ~~by~~ in GM-CSF.* This suggested that Kit could have a differentiative effect on the cells which would explain the decrease in the number of colonies formed in GM-CSF and the absence of colonies without factor.

5.3.2 Analysis of the cell types obtained following introduction of activated Kit

Cells infected with virus encoding V816Kit, WTKit or RUFneo were cultured in the absence of factor for 1 week, then examined by cytocentrifugation and staining with Giemsa. Examples from two different experiments are shown in Figure 5.5. The cells infected with WTKit virus were similar to those infected with RUFneo virus as shown in Figure 5.5 A and B. The cultures consisted mainly of dead cells with a few differentiated neutrophils and macrophages. In comparison, cultures infected with V816Kit (Figure 5.5 C and D) showed increased numbers of cells and an obvious

* see additional notes

presence of large densely staining cells. These cells are shown at a higher magnification in Figure 5.5 E and F, illustrating their large size and multiple nucleii, which are morphological features of megakaryocytes. Staining of these cells for the presence of acetylcholinesterase (section 2.2.3), an enzyme expressed by murine megakaryocytes, confirmed that they were of the megakayocytic lineage (Figure 5.6). The photographs in Figure 5.6B show megakaryocytes of various sizes and in Figure 5.6 C and D show examples of single and multi-nucleated megakaryoctes. After 1 week in culture without added factors, an increase in the number of mature cells of the GM lineages were also detected in V816Kit-infected populations (Figure 5.5 C and D), when compared to RUFneo infected poulations (Figure 5.5 A and B).

5.3.3 Effects of V816Kit and WTKit in the presence of huSCF

To determine if megakaryocytic differentiation could also be observed when WTKit was expressed in the presence of its ligand, the experiments were repeated as above, but cells infected with retrovirus encoding V816Kit, WTKit or RUFneo were cultured in the presence of huSCF. Examples of the cell types observed following culture with SCF are shown in Figure 5.7. RUFneo infected cells in the presence of SCF appeared the same as those observed in the absence of factor, i.e. mature macrophages and neutrophils as well as some dead cells (see Figure 5.5 A). However, infection with WTKit or V816Kit also resulted in the presence of megakaryocytes, as well as cells with dark staining granules marked with arrows in Figure 5.7A. These cells are shown in comparison to mature neutrophils in Figure 5.7B and a mature megakaryocyte in Figure 5.7C. Some cells displayed mature features of large size and high numbers of cytoplasmic granules as shown in Figure 5.7D. Immunocytochemical analysis of the cell populations with an anti-mast cell protease-5 antibody (anti-mMCP-5) as described in section 2.3.3B, proved the presence of mast cells within

these populations as shown in Figure 5.8. In the presence of SCF, mast cells were detected in populations infected with WTKit (panels A and C) and V816Kit (panels B and D) but not in populations infected with RUFneo (panel E). The mast cells observed were of various sizes and at various stages of differentiation, as indicated by the differences in the level of protease detected.

5.3.4 Expression of human Kit

To examine huKit expression in the populations that were infected with Kit viruses, following a week in culture the cells were analysed by APAAP as described in section 2.3.3A and the results are shown in Figure 5.9. These populations are the same as those discussed above (sections 5.2.2 and 5.3.3). RUFneo infected cells cultured in the absence of factor (Figure 5.9A) were all negative for expression as were RUFneo infected cells cultured in huSCF. The megakaryocytes were the only cell types with detectable levels of huKit among the V816Kit infected cells maintained without factor (Figure 5.9B). Most megakaryocytic cells resulting from infection with WTKit or V81Kit and culture in the presence of huSCF were also positive (Figure 5.9C), however some displayed no detectable huKit. An example of one such cell is marked with an arrow in Figure 5.9B. Since no similar cells developed in cultures of FLC infected with RUFneo virus, it is likely that these cells expressed huKit below the limit of detection. Besides megakaryocytes, another type of cell expressing huKit was observed in FLC populations infected with viruses encoding WTKit or V816Kit following culture in SCF (marked by arrows in Figure 5.9C). These cells were granular, similar to those identified as mast cells by their expression of mMCP-5 (Figure 5.8). In both the WTKit and V816Kit infected poulations, following 1 week in culture these cells were mostly small with few granules (examples are marked by arrows in Figure 5.9C) but after 2 weeks were

larger with more granular cells (examples are marked by arrows in Figure 5.9D). Following two weeks in culture with SCF megakaryoctic cells were very few in number and appeared senescent (Figure 5.9D), however mature macrophages and neutrophils were still present.

The numbers of megakaryocytes and mast cells counted after one week in different experiments are shown in Table 5.1. Mast cells were only detected in the Kit-infected populations when cultured in SCF. Analysis of cell populations at two weeks post-infection showed that in most cases there were no viable megakaryocytes present in any of the populations, but there were surviving mast cells as well as some mature macrophages and neutrophils in V816Kit infected cells cultured without factor and in V816Kit and WTKit infected cells cultured in the presence of SCF. No viable cells remained in populations infected with RUFneo by two weeks post-infection. In populations infected with virus encoding WTKit or V816Kit (Figure 5.9D) that were maintained in huSCF, by two weeks post-infection, the total number of mast cells had declined, but the proportion compared to the total number of cells had increased to approximately 25% due to attrition of the other cell types.

In all the experiments conducted, infection of FLC with virus encoding V816kit or WTkit in the presence of SCF failed to give rise to long term cell lines. Additionally, in the absence of factor, V816Kit failed to give rise to any colonies and caused a decrease in the number of colonies formed in GM-CSF when compared to the control cells (see Figure 5.4A). These results suggested that neither activated mutant Kit nor WTKit plus SCF were able to sustain the proliferation of FLC *in vitro*. Myb is a known immortalising agent in FLC. To observe any co-operative effects between oncogenes, experiments analysing the effects of co-infection with Myb and Kit were conducted.

5.4 CO-OPERATION BETWEEN MYB AND KIT IN FLC

5.4.1 Clonogenic Capacity

To determine the clonogenic capacity of FLC infected or co-infected with various viruses encoding constructs, the infected cells following co-cultivation were plated in methylcellulose containing growth factors as indicated in Figure 5.10. The assay was conducted similarly to those discussed above in section 5.3.1, except that in this experiment, some cells were co-infected with Kit viruses and CT3Myb viruses by co-cultivation with a mixed population of Ψ2 producers. The number of colonies formed by co-infected FLC are shown in Figure 5.10. FLC co-infected with V816Kit and CT3Myb gave rise to factor-independent colonies indicating that the two oncogenes were able to act co-operatively,* since infection with either alone failed to give rise to colonies in the absence of factor (see also Figure 5.4A). In the presence of GM-CSF, the V816Kit and CT3Myb co-infected FLC yielded higher numbers of colonies, but in huSCF formed fewer colonies suggesting that both factors were able to influence the clonogenicity of the infected cells. In huSCF none of the other infected FLC showed clonogenic potential. More interestingly, FLC co-infected with WTKit and CT3Myb in SCF did not have the same clonogenic capacity as FLC co-infected with V816Kit and CT3Myb in the absence of factor. This suggested that, in co-operation with activated Myb, constitutively activated mutant Kit and WTKit plus SCF exhibit different effects in haemopoietic cells. This was analysed further and will be discussed in more detail in chapter 6.

5.4.2 Proliferative Capacity

Following co-cultivation the infected FLC were also placed in liquid culture with various growth factors. The total cell numbers after different times in culture are shown in Figure 5.11A and 5.11B. As expected, FLC infected with CT3Myb and

* see additional notes

grown in GM-CSF showed sustained proliferative capacity. FLC infected with V816Kit virus did not show any increase in cell numbers at one week, but after two weeks were higher in number than control RUFneo infected FLC, suggesting that V816Kit may have a proliferative effect or is able to enhance survival. Figure 5.11B shows that FLC co-infected with V816Kit and CT3Myb, increased in number in the absence of factor. GM-CSF enhanced this increase, possibly due to the presence of cells infected with CT3Myb virus alone. SCF also enhanced the cell yield at day 7 but following that caused a decrease in cell number when compared to no added factor. These results correspond with that of the clonogenic assay shown in Figure 5.10.

The proliferative capacity of FLC co-infected with V816Kit and CT3Myb viruses and maintained for three weeks post-infection in GM-CSF (population 1), SCF (population 2), or no factor (population 3) was analysed as described in section 2.9.1A. The results in Figure 5.11C show the absorbance as a measure of cell number for the populations 1, 2 and 3 in either GM-CSF, SCF or no factor. Population 1 (that had been previously maintained in GM-CSF) was able to proliferate in GM-CSF, but not in SCF or in the absence of factor. This suggests that the majority of cells in the population expressed CT3Myb but not V816Kit, probably due to selection of the Myb infectants by maintenance in GM-CSF. Population 2 (that had been previously maintained in SCF) showed low proliferative capacity in this factor and in the absence of factor, but did proliferate in GM-CSF. Population 3 (that had been previously maintained without factor) showed the highest proliferation among all three groups in GM-CSF as well as a slight increase in cell numbers by day 6 in SCF or without added factor. Thus, this population probably contained CT3Myb and V816Kit co-infected cells that were able to proliferate in response to GM-CSF and, to a lesser

extent, without factor. The morphological characteristics of co-infected cells, cells from this were examined.

5.4.3 Morphological analysis of co-infected FLC

Examples of cells from the population of FLC co-infected with CT3Myb and V816Kit, grown in the absence of factor, are shown in Figure 5.12. The cells at week 1 resembled those infected with V816Kit alone with the presence of megakaryocytes as seen in panel A. It was unclear at this time-point as to which cells were expressing Myb or Kit. At week 2 when FLC infected with V816Kit alone were few in number with only mature neutrophils and macrophages present, the co-infected population (as seen in panel B) were heterogeneous with some immature progenitor-like cells similar to MTHC cells grown in GM-CSF (see Chapter 3 - Figure 3.6C). At week 3, the population was heterogeneous with the presence of mature myeloid cell types as shown in panel C. Cytochemical staining for expression of α-naphthyl acetate and chloro acetate esterases expressed by mature macrophages and neutrophils respectively (see section 2.2.3), confirmed the presence of maturing macrophages and a few neutrophils (Figure 5.12D) within this population. Staining for acetylcholinesterase and mast cell protease were negative and confirmed that the populations shown in Figure 5.12 B and C did not contain megakaryocytic or mast cells. In comparison, after 3 weeks in culture without factor, FLC populations infected with CT3Myb alone or V816Kit alone, contained no viable cells. As mentioned earlier, FLC infected with CT3Myb alone maintained in GM-CSF (MTHC) following 7 days in culture, are maintained as long term cell lines and consistently show features of immature progenitor cells of the GM lineages (Figure 3.5A, Figure 3.6C). The comparisons between cells expressing CT3Myb alone and CT3Myb plus V816Kit was investigated further and will be discussed in chapter 6.

Figure 5.1 : The sequence of the mutant kit clone (V816Kit) in pRUFneo.

The PCR product cloned into the pRUFneo (WTKit) plasmid was sequenced as described in section 2.5.3 using oligos 1002, SRC-05, SRC-09 and SRC-10 (see Table 2.1). The sequence is shown from nucleotide 2457 to nucleotide 2489 in the *kit* cDNA (Yarden *et al.,* 1987). The point mutation of an A to T base pair change at nucleotide 2468 is marked with an arrow on the left. The point mutation results in a change from amino acid Asp encoded by the codon GAC to amino acid Val encoded by the codon GTC at residue 816 in the human Kit protein as marked on the right.

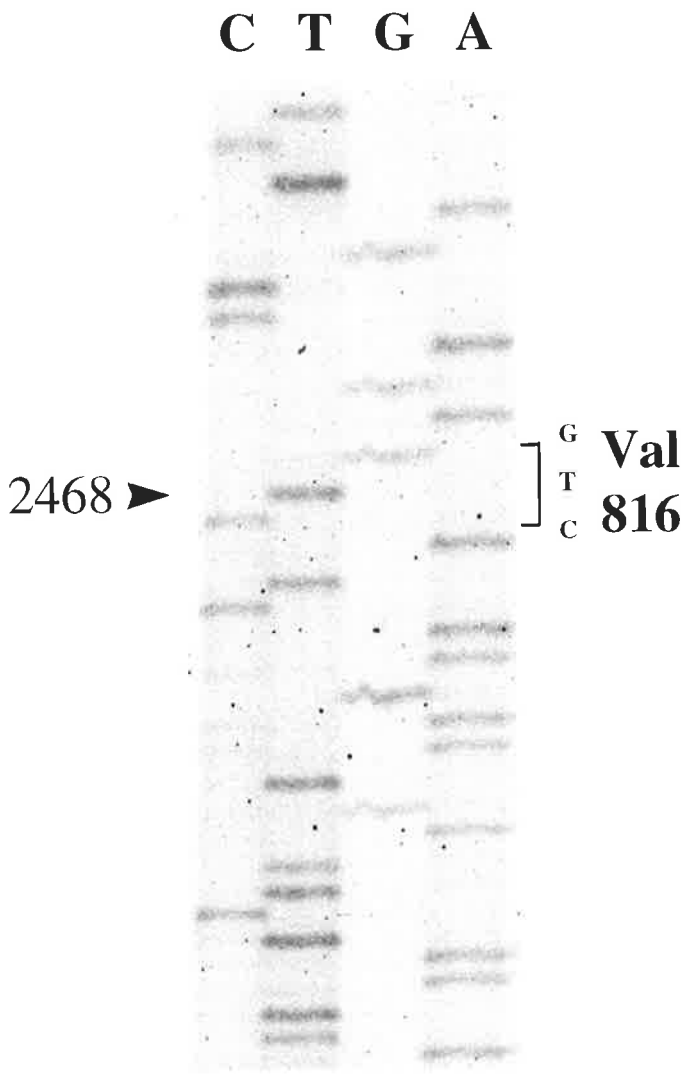

C T G A
2468
G
T
C
Val
816

Figure 5.2 : Profiles of huKit surface expression on transfected Ψ2 cells and infected FDC-P1 cells.

Immunofluorescence using 1DC3 mAb as described in section 2.3.2 was used to detect huKit expressed on the cell surface. Panel A shows the profiles of Ψ2 cells following G418 selection of RUFneo transfectants, WTKit and V816Kit sorted transfectants, as indicated. Panel B shows the profiles of FDC-P1 infectants following G418 selection. Panel C shows the profiles of the selected FDC-P1 cells following maintenance of RUFneo infectants in GM-CSF, selection of WTKit infectants by growth in huSCF and of V816Kit infectants by growth in the absence of added factor.

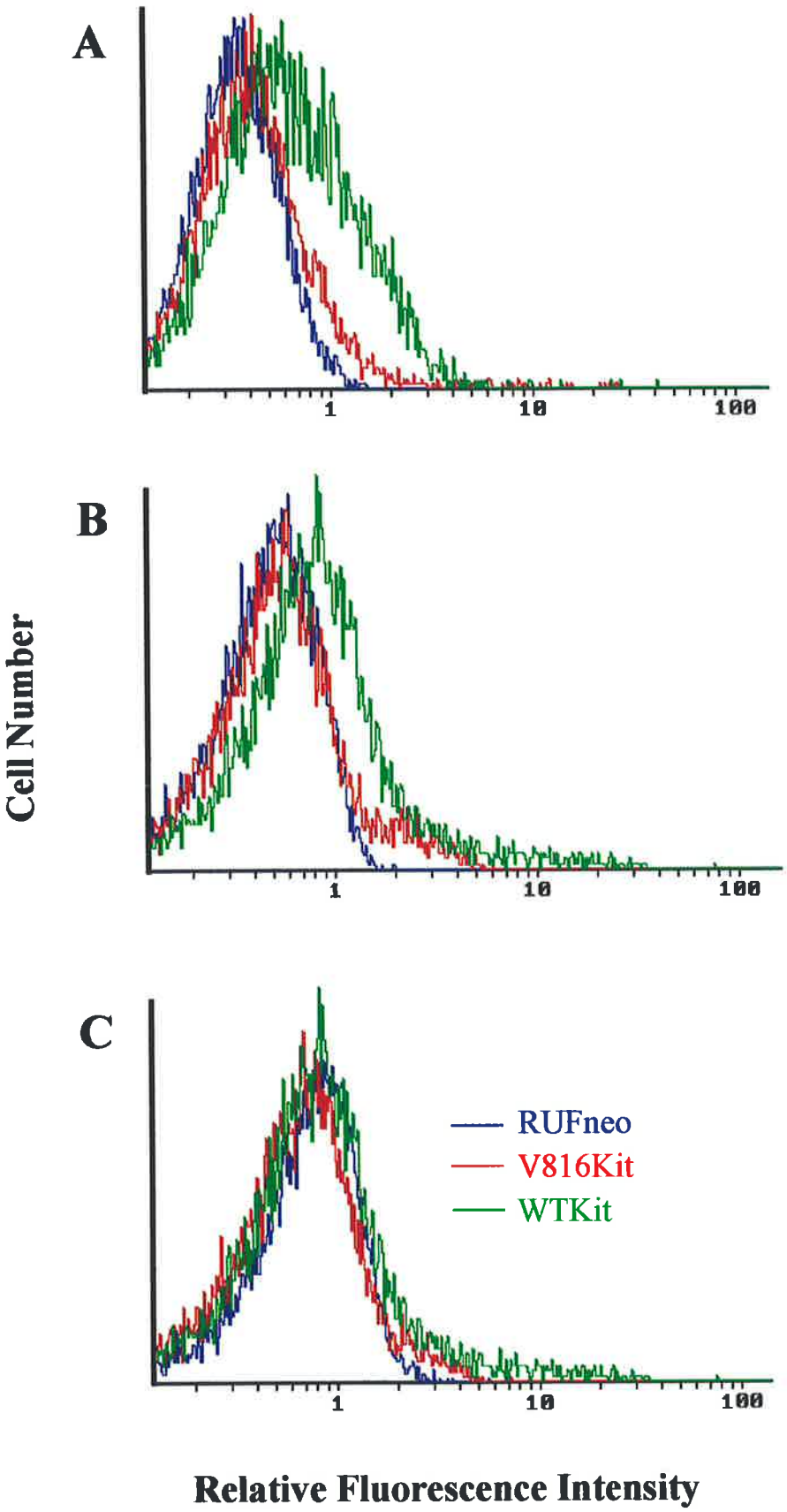

A
B
C
1
10
100
Cell Number
RUFneo
V816Kit
WTKit
Relative Fluorescence Intensity

Figure 5.3 : Colony formation by FDC-P1 infectants.

The G418 selected FDC-P1 infectants were plated in methylcellulose (as described in section 2.9.2) with GM-CSF or no added factor. The average +/- the SEM for the number of colonies formed by 2,000 cells from triplicate plates after 1 week are shown with the (*) indicating an absence of colonies.

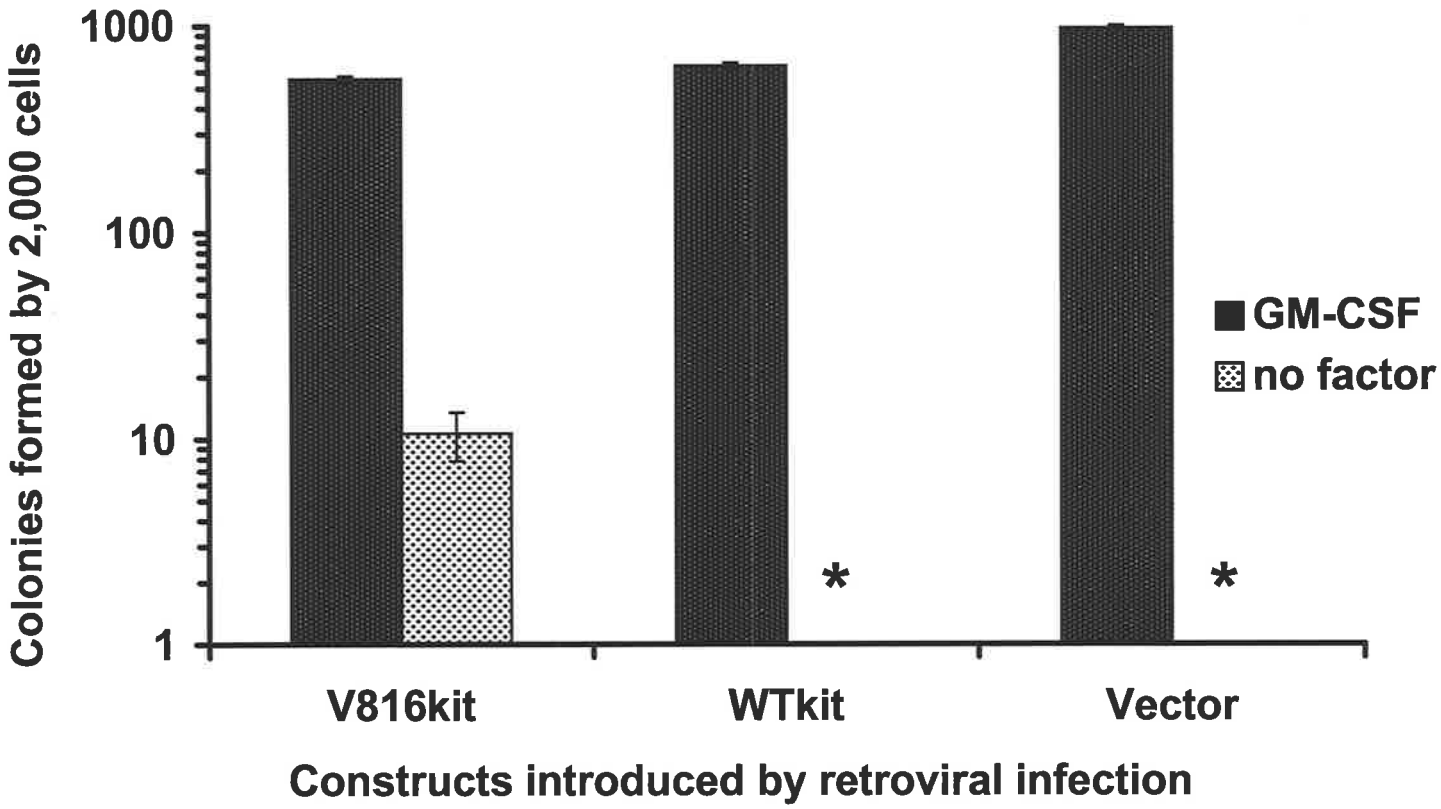

1000
100
10
1
Colonies formed by 2,000 cells
GM-CSF
no factor
*
*
V816kit
WTkit
Vector
Constructs introduced by retroviral infection

Figure 5.4 : Colony formation by FLC infected with virus encoding WTKit or V816Kit.

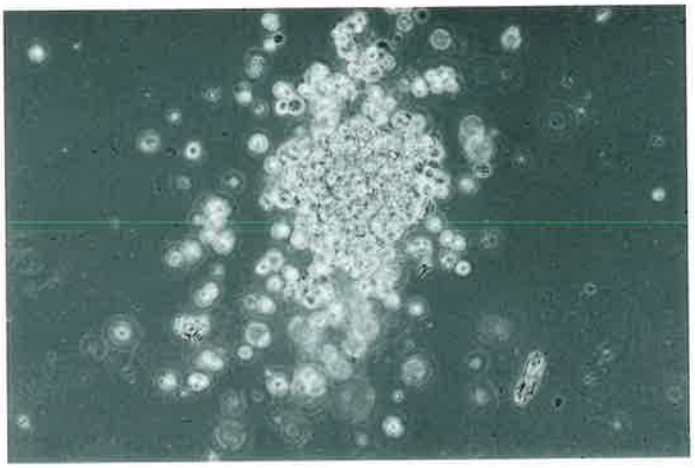
Following infection with the retroviral constructs as described in section 2.8.3B, FLC were assayed for clonogenicity in GM-CSF or in the absence of added factor, as indicated. The results in Table A show the average +/- the SEM of triplicates for the number of colonies formed after 14 days by $1x10^4$ cells plated/dish. Panel B shows an example of a colony formed by V816Kit infected FLC in GM-CSF, which is similar to those observed for the other FLC plated in GM-CSF. This is a representative example of the colonies counted (containing more than 50 cells). Panel C shows an example of a 'cluster' of cells (containing 10-25 cells) formed by V816Kit infected FLC in the absence of factor. The clusters were not scored as colonies in Table A. The frequency of clusters detected ranged from 0-5 per dish with an average of 1.6, 2.0 and 0.6 for the three separate experiments respectively.

The colonies shown in panels B and C were initially photographed at a magnification of 20x.

A

	Experiment 1	Experiment 2	Experiment 3
GM-CSF			
V816Kit	9.0 +/- 1.4	3.7 +/- 1.1	0.7 +/- 0.4
WTKit	12.0 +/- 1.2	2.7 +/- 1.1	4.7 +/- 2.2
CT3Myb	114.0 +/- 9.4	450.7 +/- 30.2	372.0 +/- 10.5
RUFneo	18.0 +/- 0.7	10.0 +/- 3.1	ND
no Factor			
V816Kit	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0
WTKit	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0
CT3Myb	0.0 +/- 0.0	0.0 +/- 0.0	0.0 +/- 0.0
RUFneo	0.0 +/- 0.0	0.0 +/- 0.0	ND

B



C

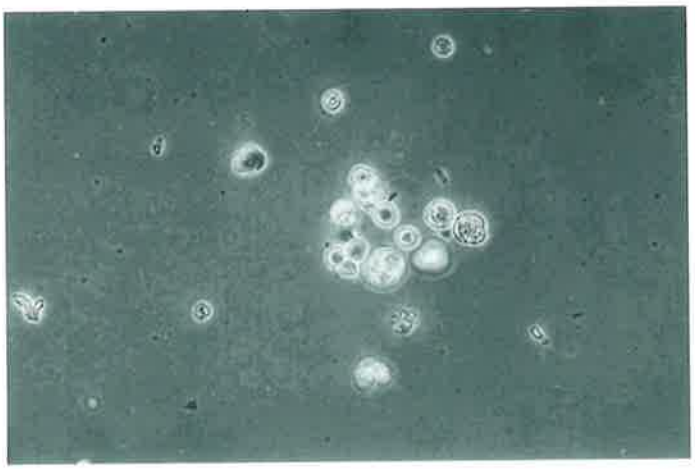


Figure 5.5 : Morphology of FLC infected with virus encoding vector (RUFneo) or RUFneo(V816Kit).

Photographs show representative cells from the populations following cytocentrifugation and Wright-Giemsa staining. The two columns show infected FLC following 1 week of culture in the absence of factor for two different experiments. Panels A and B represent RUFneo infected control FLC and panels C and D represent V816Kit infected FLC, all photographed at 10x magnification. Panels E and F represent the same V816Kit infected FLC, photographed at 20x magnifaction.

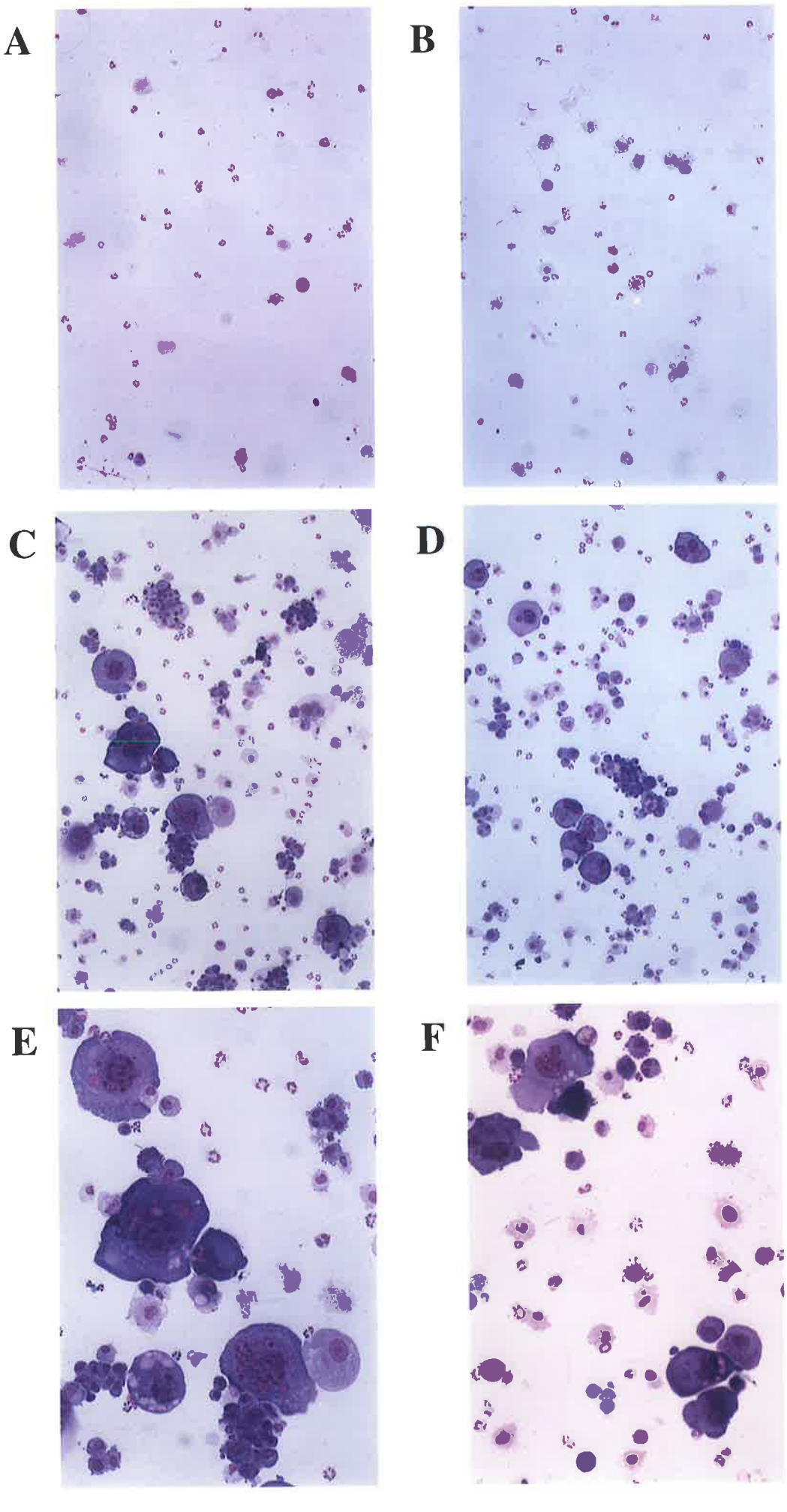

A
B
C
D
E
F

Figure 5.6 : Expression of acetylcholinesterase by FLC infected populations.

The Photographs show cell smears (as for Figure 5.5) stained for the presence of the megakaryocytic enzyme acetylcholinesterase. The red brown precipitate indicates enzyme activity and the blue staining is derived from a haematoxylin counterstain. The photographs show representative infected FLC derived after 1 week in culture without factor. Panel A and B show the results from a single experiment of RUFneo-infected FLC and V816Kit-infected FLC respectively, photographed at 10x magnifucation. Panels C and D show V816Kit-infected FLC from two different experiments photographed at 40x magnification.

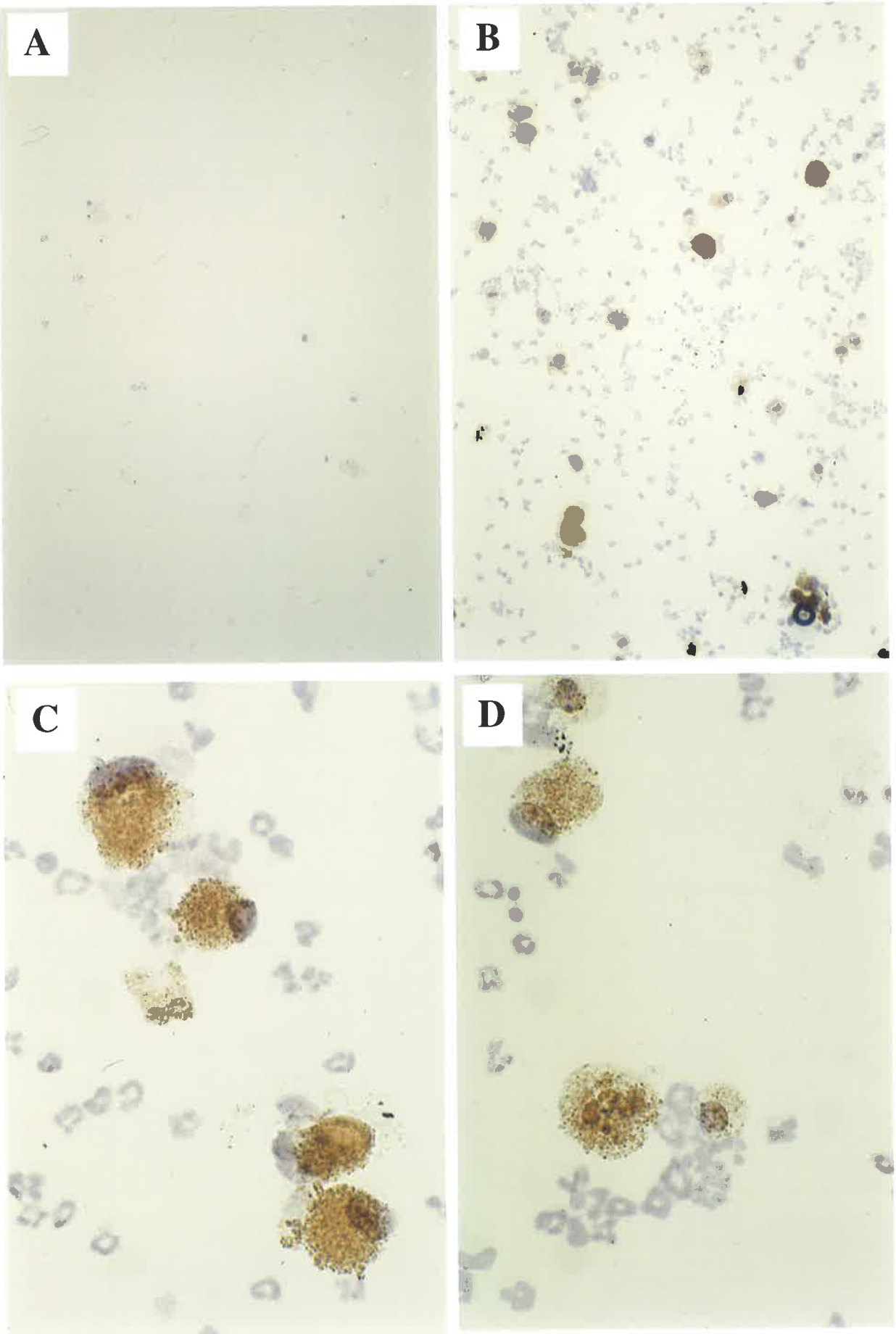

A
B
C
D

Figure 5.7 : Morphology of infected FLC cutured in SCF.

Photographs show representative cells from the total populations following cytocentrifugation and Wright-Giemsa staining. The panels show infected FLC following one week of culture in the presence of huSCF, with the two columns showing results from two different experiments. Panels A and B show WTKit-infected FLC, photographed at 20x and 40x magnification respectively. Panels C and D show V816Kit-infected FLC photographed at 40x magnification. The arrows indicate the 'granular' cells detected in the different populations.

RUFneo infected (control) FLC maintained in SCF were identical to those maintained in the absence of factor (Figure 5.5 A and B)

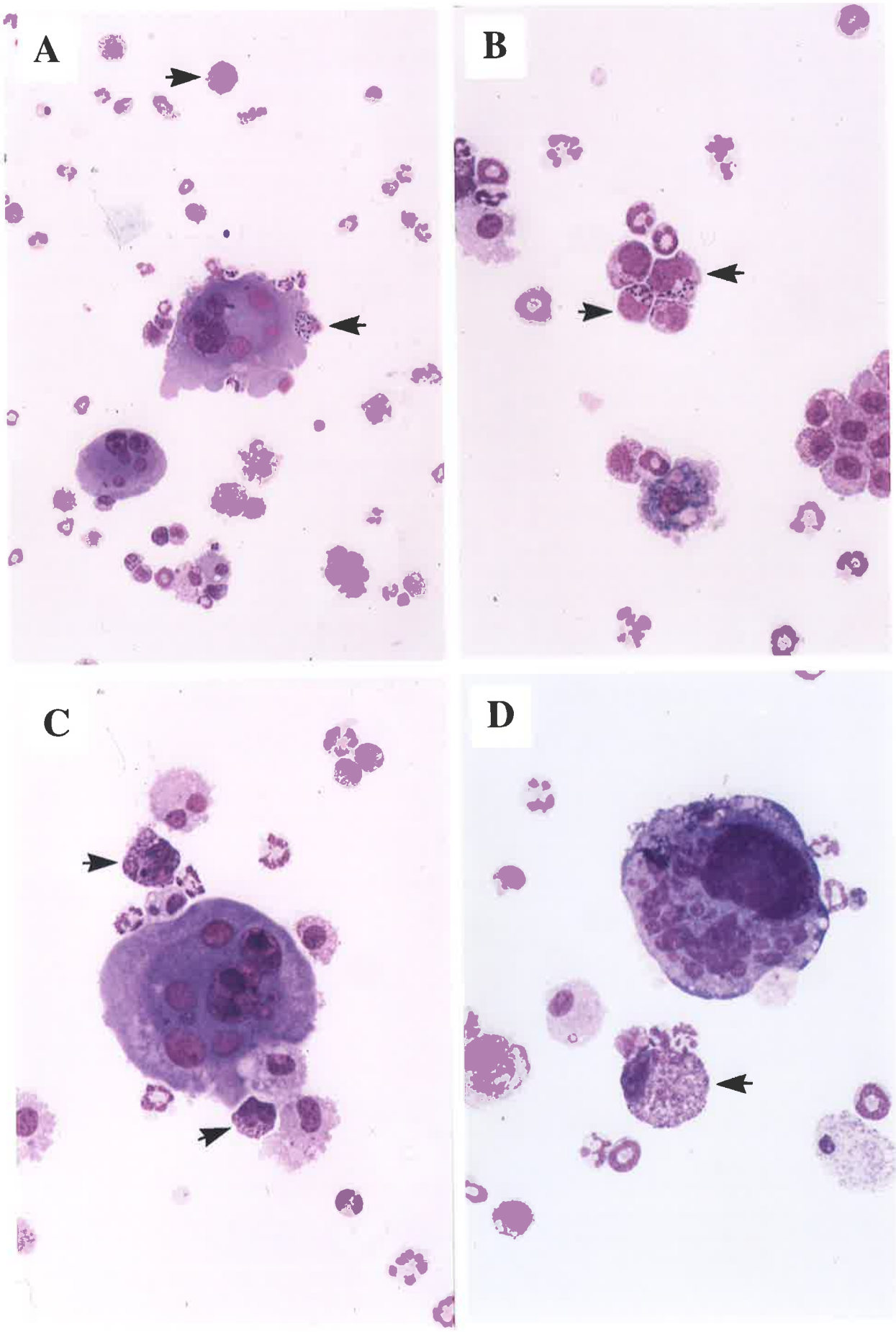

A
B
C
D

Figure 5.8 : Immunocytochemical staining for murine mast cell protease-5.

The cells shown are representative of infected FLC from one experiment following one week of culture in huSCF, that were analysed by APAAP for the expression of MCP-5. The intense red staining shows granules containing the protease and the blue staining is derived from a haematoxylin counterstain. Panels A and B show WTKit and V816Kit infected FLC photographed at 10x magnification. Panels C and D show different fields of view of the same cell populations photographed at 20x magnification. Panel E shows control RUFneo-infected FLC photographed at 10x magnification.

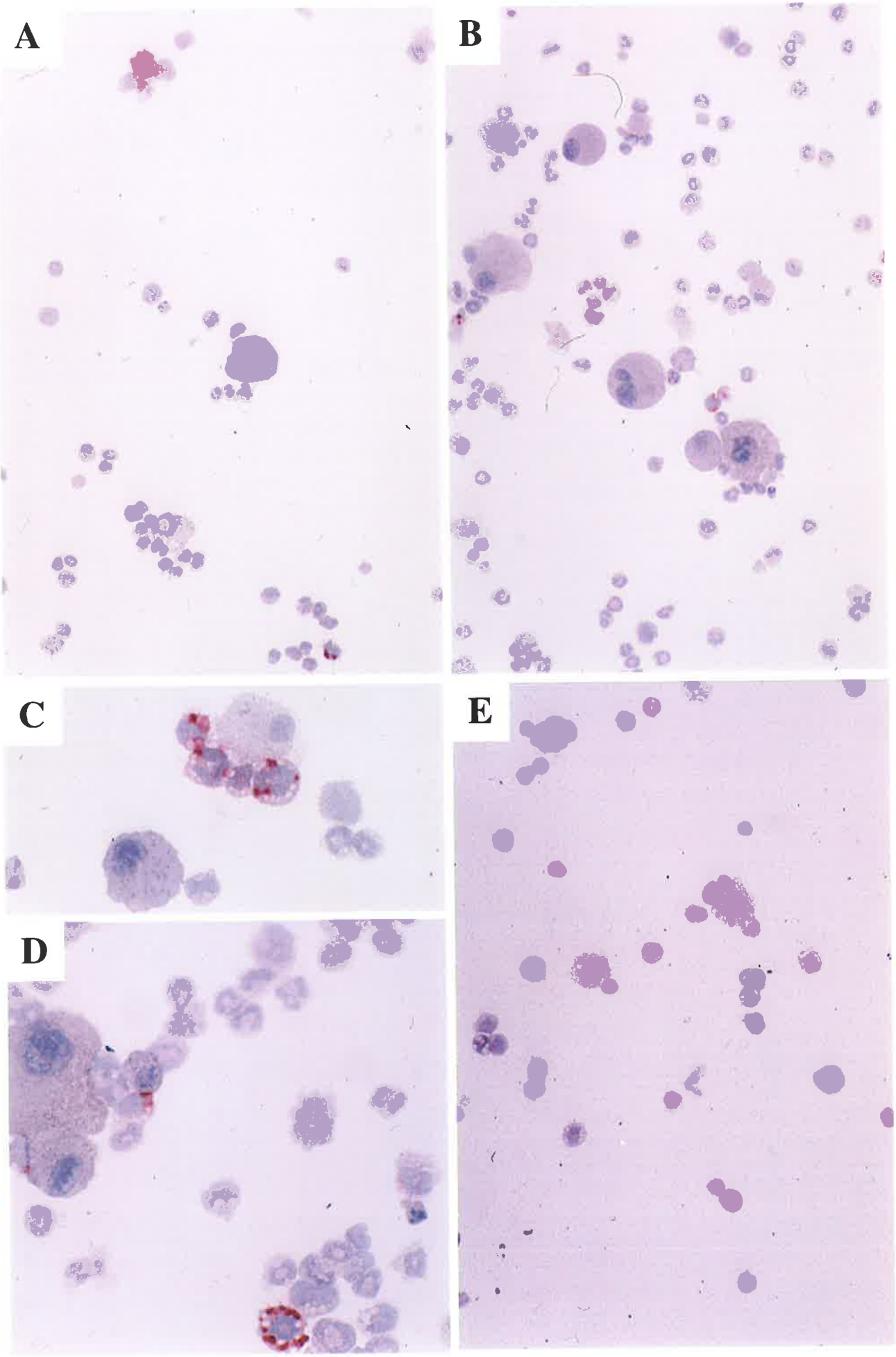

A
B
C
D
E

Figure 5.9 : Photographs of immunocytochemical staining by the APAAP method for human Kit with mAb 1DC3.

The red staining indicates the presence of human Kit protein and the blue staining is derived from a haematoxylin counterstain. The cells shown are representative of infected FLC from a single experiment. Panel A shows RUFneo infected FLC after 1 week in culture with huSCF, the cells shown being similar to those observed when cultured without factor. Panel B shows V816Kit infected FLC after 1 week in culture without factor. Panel C shows WTKit infected FLC after 1 week in culture with huSCF. Panel D shows V816Kit infected FLC after 2 weeks in culture with huSCF. All panels were photographed at 10x magnification.

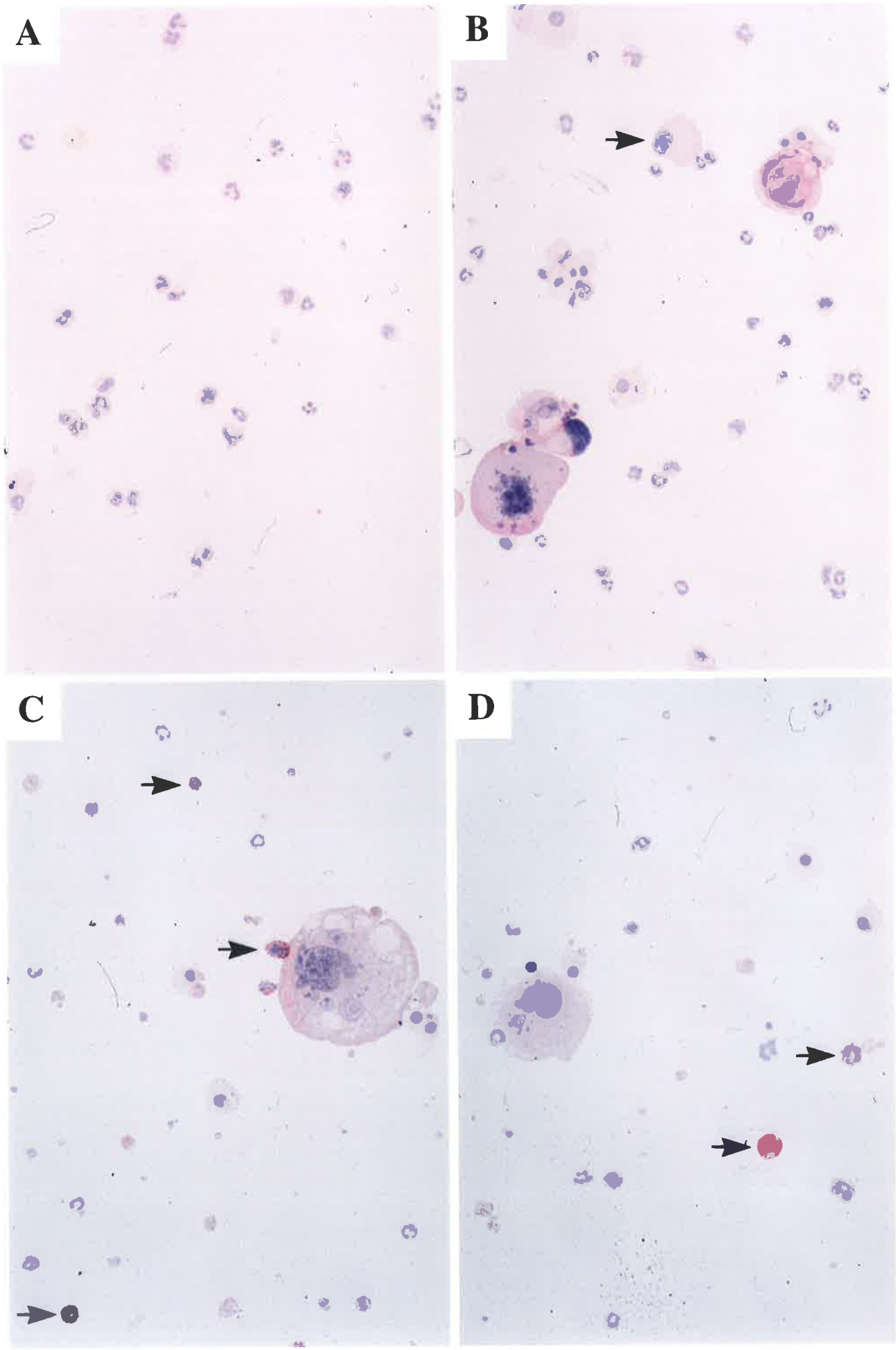

A
B
C
D

Table 5.1 : Proportions of specific cell types in FLC populations following 1 week in culture.

The number of megakaryocytes and mast cells per 1000 cells counted are shown in Table A and Table B respectively. The constructs introduced by infection and the growth factors added during culture are indicated in each table. The results of 6 different experiments are presented. The numbers shown were derived by counting 750-2000 cells (depending on the total number of cells in each populatien) on cytocentrifuge smears that were stained with Giemsa and/or analysed for expression of acetylcholinesterase or expression of mMCP-5. 'nd' indicates not done.

A

	Number of megakaryocytes / 1,000 cells					
	no factor			huSCF		
	RUFneo	V816Kit	WTKit	RUFneo	V816Kit	WTKit
Experiment 1	0	185	0	nd	nd	nd
Experiment 2	0	173	nd	nd	nd	nd
Experiment 3	1	239	nd	1	58	nd
Experiment 4	0	136	nd	0.3	46	nd
Experiment 5	0	57	0	0	27	26
Experiment 6	2	185	0	0.6	70	86

B

	Number of mast cells / 1,000 cells			
	huSCF			no factor
	RUFneo	V816Kit	WTKit	V816Kit
Experiment 3	5.4	27.5	nd	0
Experiment 4	0	4	nd	0
Experiment 5	0	10.9	9.7	0
Experiment 6	3	8.4	8.8	0

Figure 5.10 : Colony Assay of FLC infected or co-infected with WTKit, V816Kit and/or CT3Myb encoding viruses.

FLC were infected by co-cultivation with 10^6 Ψ2 cells or co-infected by co-cultivation with a mixture of $5x10^5$ cells of each Ψ2 producer line as indicated. The infected FLC were plated at $2x10^4$ cells/dish in methylcellulose with the added growth factors muGM-CSF (GM), huSCF (SCF) or no added factor (none). The graph shows the ~~average~~ number of colonies per dish counted after 14 days as the average +/- standard error for triplicate cultures.

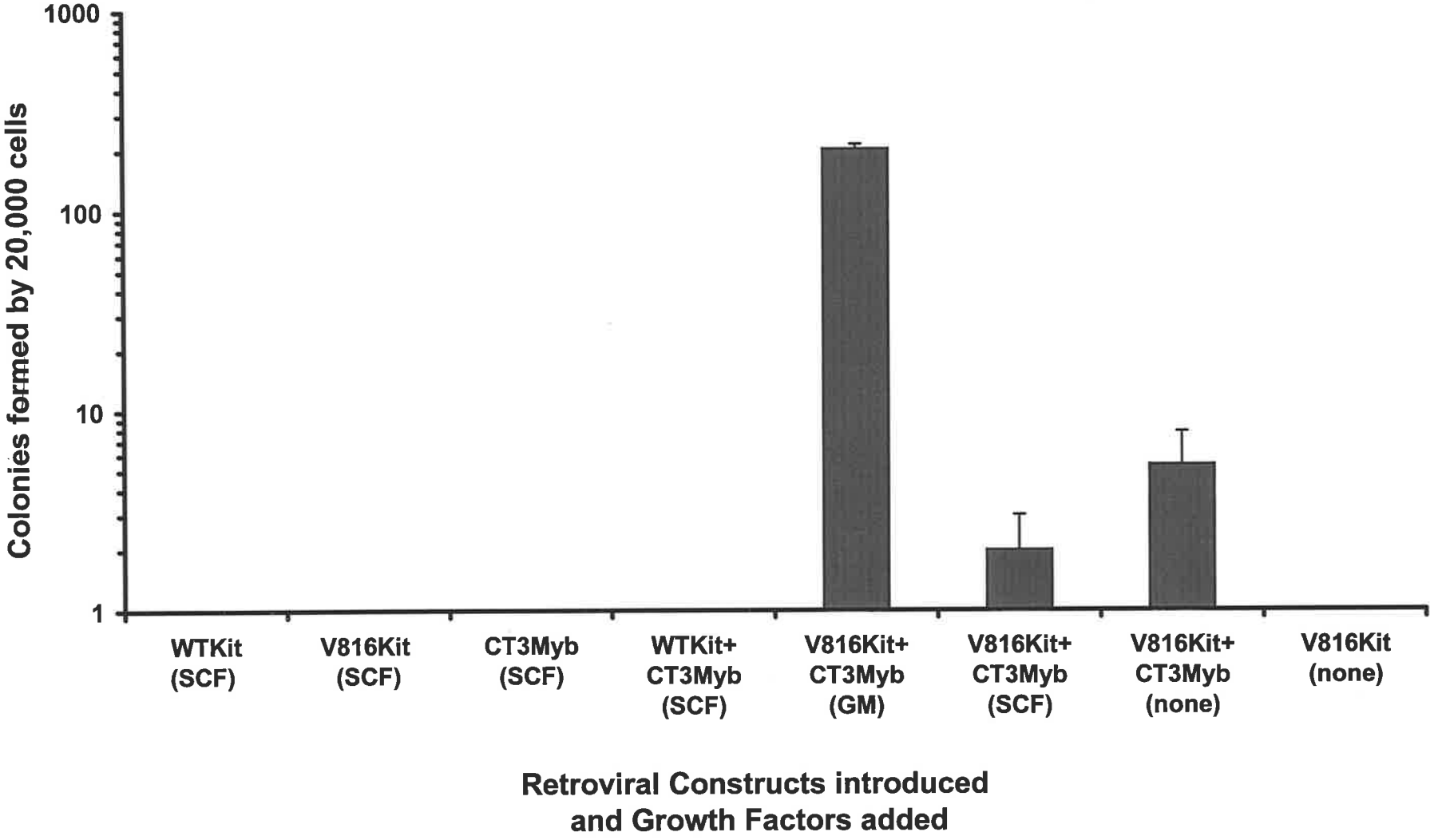

1000
100
10
1
Colonies formed by 20,000 cells
WTKit (SCF)
V816Kit (SCF)
CT3Myb (SCF)
WTKit+ CT3Myb (SCF)
V816Kit+ CT3Myb (GM)
V816Kit+ CT3Myb (SCF)
V816Kit+ CT3Myb (none)
V816Kit (none)
Retroviral Constructs introduced and Growth Factors added

Figure 5.11 : Growth charateristics of infected and co-infected FLC.

FLC transduced with the indicated retrovirus construct as described in Figure 5.10 were cultured for up to 3 weeks in GM-CSF, SCF or without factor (none).

Tables A and B show the number of viable cells (determined by counting cells that excluded trypan blue while using a haemocytometer) that were present in infected and co-infected FLC populations respectively at different times following infection.

Graphs 1, 2 and 3 in Part C show the results of a proliferation assay conducted on FLC co-infected with V816Kit and CT3Myb that had been grown for 3 weeks in GM-CSF, SCF or no factor respectively. The total cell numbers in the cultures were analysed at 2, 4 and 6 days by a proliferation assay (see section 2.9.1A) and the results are represented as the Absorbance

A

Construct GF	Cell Yield (x 10,000) RUFneo none	V816Kit SCF	V816Kit none	CT3Myb GM-CSF	CT3Myb none
7 days	1.6	1.1	1.3	92.3	2.6
14 days	0.11	0.49	0.53	125	0.52
21 days	0.02	0.016	0.018	nd	0.014

B

Construct GF	Cell Yield (x 10,000) V816 + CT3 GM-CSF	V816 + CT3 SCF	V816 + CT3 none
7 days	69.2	75	24.3
11 days	76	17	22
14 days	83	16	28
18 days	100	15	26
21 days	302	16	50

C

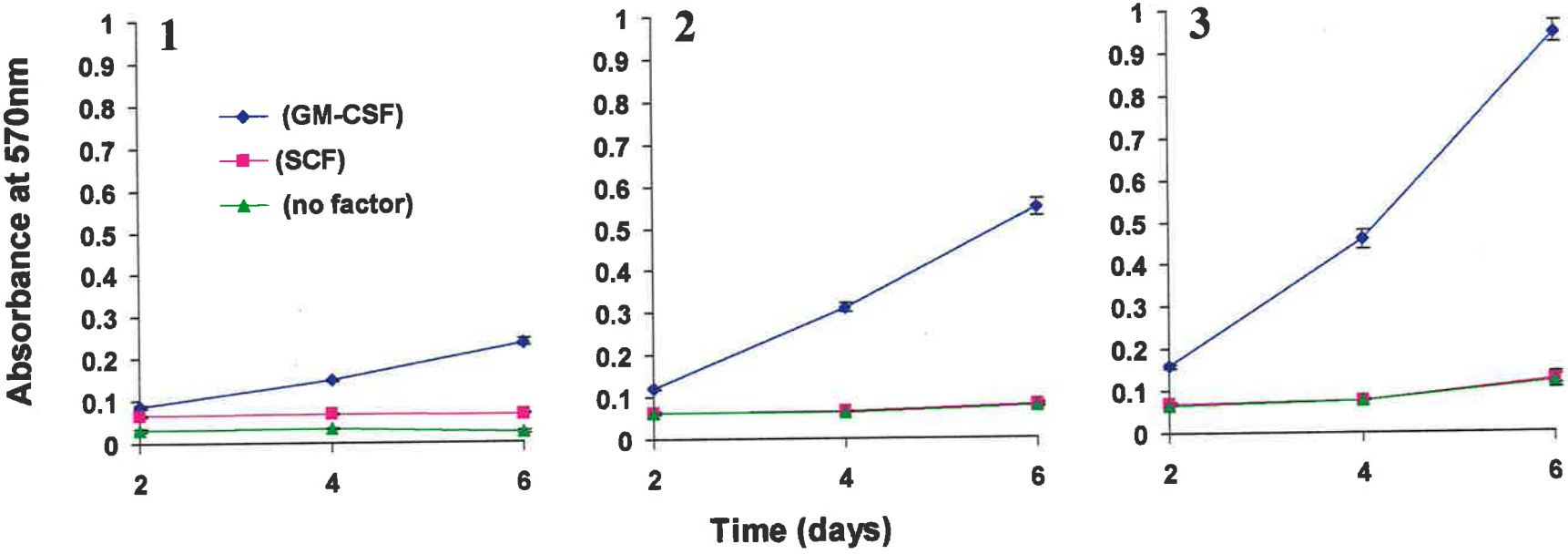
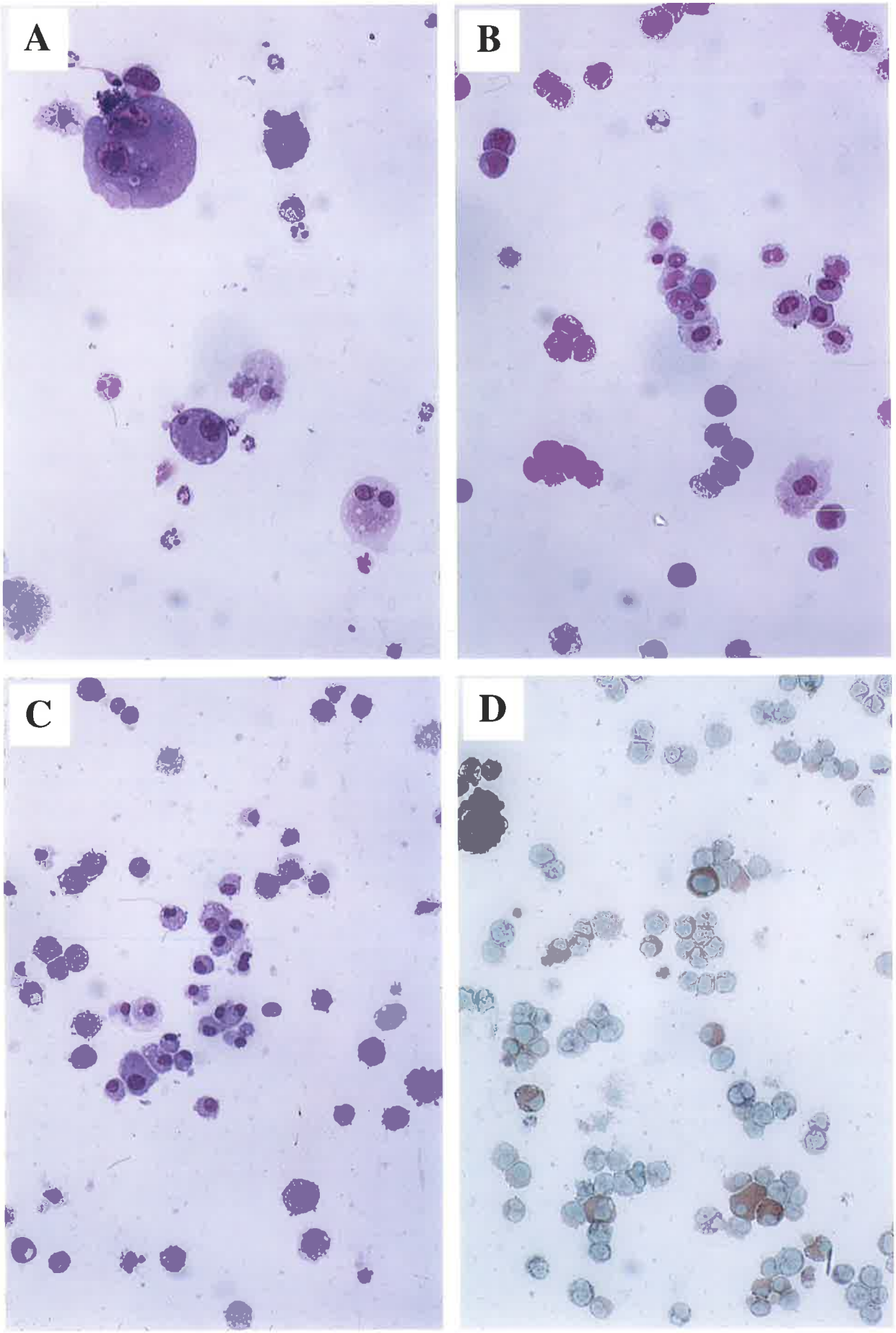


Figure 5.12 : The cellular phenotypes of FLC co-infected with V816Kit and CT3Myb.

Co-infected FLC were maintained in culture without growth factors and the cell populations were analysed for up to 3 weeks. The photographs shown are of representative cells at 20x magnification. Panels A, B and C show Wright-Giemsa stained cytocentrifuge smears of the population following 1, 2 and 3 weeks in culture post-infection respectively. Panel D shows the expression of esterases by the same population as in panel C (after 3 weeks in culture). The cytoplasmic red staining is indicative of α-naphthyl acetate (macrophage) esterase, cytoplasmic blue staining is indicative of naphtol-AS-D-chloroacetate (neutrophil) esterase, and the green staining is derived from a methyl green counterstain.


A
B
C
D

5.5 DISCUSSION

The ultimate aim of this work was to determine the co-operative effects of Myb and Kit, but firstly to determine the effects of each individually. The effects of activated forms of Myb in primary haemopoietic cells from foetal liver have been well documented as discussed in chapter 1 and chapter 3. Briefly, enforced expression of Myb in FLC, gives rise to early myeloid cells that display clonogenic potential while maintained in GM-CSF. One of the aims of the experiments discussed in chapter, was to analyse the effects of activated V816Kit in FLC.

Constructs of human WTKit and V816Kit in pRUFneo, introduced into FDC-P1 cells was able to confer growth in huSCF and in the absence of factor respectively (Figure 5.3). This effect was similar to that observed in a previous study (Kitayama *et al.,* 1995) and confirmed that the two forms of Kit were expressed from the constructs and were functional.

Colony assays (see Figure 5.4) showed that introduction of V816Kit into FLC plated at 2 x 10^4/dish only gave rise to a few clusters (on average 1.4) of mainly differentiated cells in the absence of factor. BM cells infected with the corresponding murine mutant form of activated Kit ($KITV^{814}$) were shown to form colonies (between 6-18) in the absence of factor, however this was at a comparitively higher cell density of 1.5 x 10^5 /dish (Kitayama *et al.,* 1996) and the number of cells per colony counted is not given. The number of clusters formed by V816Kit-infected FLC in the results presented here are only slightly lower than the number of colonies formed by the equivalent amount of V814Kit-infected BM cells in the study mentioned above (Kitayayma *et al.,* 1996). This could be due to the differences in the density of cells plated, since haemopietic cells are known to produce stimulatory growth factors. A similar finding between the two studies was that activated Kit decreased the

clonogenic capacity of haemopoietic cells in the presence of growth factor when compared to control cells, suggesting a decrease in their proliferative potential. It is possible that Kit could be involved in inducing differentiation of primary cells, an effect shown to occur in mast cell line IC-2 (Hashimoto *et al.,* 1996).

Analysis of the cell lineages present in cultures of FLC infected with V816Kit virus in the absence of factor or FLC infected with WTKit virus in the presence of SCF (Figure 5.5 and 5.7, Table 5.1), showed that activation of Kit led to the expansion of cells of the megakaryocytic lineage that were positive for expression of human Kit (Figure 5.9). Small numbers of mast cells were also obtained from populations infected with WTKit and V816Kit virus in the presence of SCF (Figure 5.7 and 5.8, Table 5.1) up to 2 weeks following infection. These data indicate that enforced expression and activation of Kit can result in the production of megakayocytes and mast cells from primary haemopoietic cells. It is interesting that the development of mast cells from cells infected with V816Kit virus required the presence of SCF, indicating that mutant V816Kit does not transduce a 'complete' signal in the absence of ligand. The presence of megakaryocytic and mast cells in the Kit-expressing cultures corresponds with the cell types detected in the BM of patients that carried the V816 mutation in Kit (Nagata *et al.,* 1995). All four patients in that study displayed diffuse mast cell lesions, increased numbers of megakaryocytes and dysplastic features in cells of the myeloid, megakaryocyte or erythriod lineages (Nagata *et al.,* 1995).

In normal haemopoiesis, it has been shown that c-Kit is expressed on human megakaryocytes and that SCF in combination with GM-CSF, IL-3 or IL-6 incresed the formation of megakaryocyte progenitor colony formation (Avraham *et al.,* 1992a). Also, the membrane bound form of SCF expressed on BM stromal fibroblasts has

been shown to increase adhesion and proliferation of megakaryoctes (Avraham *et al.*, 1992b). The expression of c-Kit has also been detected at high levels on mature human mast cells (Mayrhofer *et al.*, 1987) and SCF promotes the differentiation of mast cell progenitors derived from human foetal liver (Irani *et al.*, 1992), cord blood and peripheral blood (Valent *et al.*, 1992). Taken together this suggests that c-Kit plays an important role in normal production of megakaryocytes and mast cells, and that enforced expression of either activated mutant V816Kit or WTKit (in the presence of SCF), can cause increased production of these types of cells.

The results in Figure 5.5 showed that in populations infected with V816Kit virus, cells of the granulocyte and macrophage lineage were observed at higher numbers when compared to controls. However, expression of human Kit was not detected in cells from these lineages (Figure 5.9). In view of the expression levels observed on factor-independent cells known to express functional V816Kit (Figure 5.2 B and C), it was unclear if some of these cells were infected with Kit virus and expressing low levels of protein or were 'by-stander' cells expanded as a result of factors produced by other cells in the population. In the study discussed previously (Kitayama *et al.*, 1996), activated murine Kit expressed in BM gave rise to factor-independent CFU-mix (containing a mix cells from granulocyte/ macrophage/ megakaryocyte/ mast or erythroid lineages). Cells from only 2 of 5 CFU-mix were maintained for 5 weeks in culture and after 2 weeks they displayed features of mast cells (Kitayama *et al.*, 1996). Together these data all suggest that the predominant effect of activated Kit is the production of mature mast cells, with an increase in megakaryocytes, indicating a survival and differentiative effect on these lineages.

FLC co-infected with V816Kit and CT3Myb virus were able to form factor-independent colonies (Figure 5.10) and to grow in liquid culture without factor

(Figure 5.11B) indicating that Myb and Kit were able to act co-operatively. Additionally, GM-CSF was able to enhance the growth of co-infected FLC (Figure 5.10, 5.11B) but huSCF had a negative effect on growth (Figure 5.10, 5.11B). The effects of these factors in particular was analysed further and is discussed in Chapter 6. Following growth in culture without factor for 3 weeks, V816Kit and CT3Myb co-infected FLC showed phenotypic characteristics of early and mature myeloid cells of the GM lineage (Figure 5.12). Since, CT3Myb is known to block cells in an immature state, the production of mature myeloid cells, suggest that V816Kit is able to induce differentiation. This was investigated further and is discussed in chapter 6. Additionally, the proliferative and clonogenic results in this chapter seem to suggest that WTKit plus SCF did not have the same effect as V816Kit in co-operation with CT3Myb, which was also investigated further and is discussed in Chapter 6.

Chapter 6 : CO-OPERATIVITY BETWEEN CONSTITUTIVELY ACTIVATED HUMAN KIT AND ACTIVATED MYB.

6.1 INTRODUCTION

Our experiments detailed in the previous chapter showed that introduction of either activated Myb alone or activated Kit alone in murine primary haemopoieticcells failed bring about growth of factor independent cell lines or colonies, although V816Kit did promote the production of megakaryocytes and mast cells in short term cultures. However, the two oncogenes in synergy were able to give rise to factor independent cell lines in culture and factor independent colonies in methylcellulose. These data suggest that it is possible to obtain 'complete' transformation of primary cells by co-expressing activated forms of Kit and Myb in haemopoietic cells. Our interest was in determining the effects of c-Kit as well as constitutively activated (V816) Kit in synergy with the *myb* oncogene. In preliminary experiments in which FLC were co-infected with retroviruses encoding CT3Myb and WTKit, neither colonies nor cell lines were obtained in SCF-containing cultures (Chapter 5). Therefore, in order to compare the effects of V816Kit with that of WTKit, FLC lines derived from infection with CT3Myb that had been maintained in GM-CSF, were super-infected with Kit viruses.

Expression of constitutively activated Kit was shown previously to result in factor independence and tumourigenicity of myelomonocytic FDC-P1 cells (Kitayama *et al.,* 1995; Figure 5.3), and to induce a differentiated phenotype in the IC-2 mast cell line (Hashimoto *et al.,* 1996). MTHC represent a good model for investigating the effcts of activated Kit for several reasons. Like FDC-P1, MTHC lines are factor dependent, show characteristics of early myeloid progenitor cells, are highly proliferative and clonogenic (see chapter 3). It has already been shown that

expression of WTKit or V816Kit in FDC-P1 cells was able to confer growth in huSCF and in the absence of factor respectively (section 5.2.3). If MTHC behave in a similar manner, this would enable a comparison of the effects of WTKit (plus SCF) to V816Kit. It has also been shown that muSCF alone was unable to sustain clonogenicity of MTHC (that usually express low levels of murine c-Kit), but displayed synergistic effects on growth in combination with GM-CSF and IL-3 (Gonda *et al.,* 1993). This effect was similar to that observed on normal haemopoietic progenitor cells (reviewed in Broxmeyer *et al.,* 1991b), implying that MTHC could also respond to Kit-generated signals. Additionally, unlike some other cell lines for which the oncogenic factors are not yet defined, MTHC have been immortalized by a known oncogene, namely Myb. This makes them ideal for determining the co-operative effects of Myb and Kit. Futhermore, the responses of MTHC to various growth factors with proliferative and differentiative effects has been previously studied (Gonda *et al.,* 1993) and, in response to TNF-α plus IL-4, MTHC were capable of differentiating to mature macrophages and to a lesser extent mature neutrophils (Gonda *et al.,* 1993). Hence any differentiation-inducing effects of Kit could also be observed in these cells. Furthermore, MTHC have also been shown to become tumourigenic after factor-independence is acquired or enforced (Gonda *et al.,* 1989a). Thus these cells provide an excellent model for investigating the effects of WTKit and activated V816Kit on survival, proliferation, differentiation and tumourigenicity of early myeloid haemopoietic cells.

6.2 GENERATION OF KIT-EXPRESSING MTHC

MTHC infected with retroviruses encoding wild type human c-Kit (WTKit) and the mutant form containing an Asp to Val substitution at residue 816 (V816Kit)

were selected in medium containing G418 and then plated in methylcellulose with GM-CSF or without factor. The numbers of colonies obtained after 1 week are shown in Figure 6.1. As expected, parental MTHC and WTKit cells were unable to grow in the absence of exogenous growth factor, however the V816Kit cells were able to form colonies, albeit at a 50-fold lower frequency than parental cells in GM-CSF, indicating that some of these cells were factor independent.

The cell populations were selected to obtain cells that were all expressing Kit. The WTKit cells were isolated by fluorescence activated cell sorting based on surface expression of human c-Kit and then selected for growth in huSCF. The V816Kit cells were directly selected for growth in liquid culture in the absence of growth factor, since the level of surface expression of hu c-Kit was low (see below, Figure 6.2). All further experiments involved parental MTHC maintained in murine GM-CSF, WTKit cells maintained in human SCF and V816Kit cells maintained in the absence of added factor.

Cell surface expression of human Kit by the selected cells as shown in Figure 6.2(A) was determined by flow cytometry using mAb 1DC3 as described in section 2.3.2. Human Kit was expressed by all WTKit cells but was not detectable on the surface of V816Kit cells. Low surface expression of this mutant form of murine Kit was also observed in other cell types (Kitayama *et al.,* 1995) and was thought to be due to its down-regulation caused by continuous degradation (Moriyama *et al.,* 1996). A low level of surface expression of constitutively phosphorylated mutant c-Fms receptors, again due to receptor degradation, has also been previously observed (Glover *et al.,* 1995). It is therefore likely that our failure to detect V816Kit surface expression was due to downregulation caused by degradation of V816Kit in MTHC. Despite the fact that the V816Kit cells did not express any detectable cell surface

human Kit, they were able to survive without factor, unlike parental MTHC and WTKit MTHC. To confirm the expression of human Kit in these cells, immunocytochemical staining as described in section 2.3.3A was used to detect both cell surface and intracellular Kit protein. The results of immuno-staining with mAb 1DC3 using the APAAP method of detection for total human Kit protein expression are shown in Figure 6.2(B) The parental cells did not express human c-Kit (the light staining is background). The WTKit cells have high levels of total Kit protein, but the V816Kit cells have low and in some cases undetectable levels which is again consistent with rapid turnover of activated Kit protein.

6.3 PROLIFERATIVE CAPACITY OF KIT EXPRESSING MTHC

The growth rates, the average number of cell divisions, and the survival of the selected cell populations in liquid culture were analysed as described in section 2.9.1B and the results are shown in Figure 6.3A-C, 6.3D-F and 6.3G-I respectively. Like the parental MTHC, the Kit-expressing cells grew in the presence of murine GM-CSF, however the V816Kit cell population increased at a faster rate than the parental MTHC, while WTKit cells grew at a relatively slower rate (Figure 6.3A). A possible reason for this is the change in growth factor as the cells were previously maintained in SCF; although it is probably more likely to be a reflection of prior differentiation of this cell group in SCF (see below and Figure 6.7). The average number of divisions of the three groups over the course of the assay, were very similar (Figure 6.3D), so the variation in cell number was primarily due to the differences in the survival of these cells (Figure 6.3G). No death of V816Kit cells was seen over the four day culture period, while considerable turnover of WTKit and the parental cells was observed. The latter could reflect the requirement for other autocrine factors

(MacMillan and Gonda 1994) in the culture medium which were removed by washing the cells prior to assay. In medium containing human SCF the parental MTHC did not survive (Figure 6.3H) while the WTKit cells proliferated (Figure 6.3B) but showed rapid turnover, as in GM-CSF (Figure 6.3H). Figure 6.3C, 6.3F and 6.3I show that in the absence of growth factors only the V816Kit cells were able to survive and proliferate. The growth rate of these cells was higher in presence of GM-CSF than without added factors and GM-CSF could to some extent enhance their proliferation and survival. However it is important to note that the V816Kit cell populations always doubled at a higher rate than the other cell populations (including the parental cells).

6.4 CLONOGENIC CAPACITY OF KIT EXPRESSING MTHC

6.4.1 Colonies formed by the different cells in various factors

The Kit-expressing cell populations in liquid cultures were heterogeneous and contained more adherent cells than the parental population; hence, the adherent and non-adherent cells were analysed separately in colony assay as described in 2.9.2. Figure 6.4 shows that the parental MTHC were not able grow in semi-solid medium with SCF or without factor and the WTKit cells were unable to grow without factor as was the case in liquid culture (see Figure 6.3). In GM-CSF, the V816Kit cells formed more colonies and the WTKit cells less colonies than the parental cells. The number of colonies formed in all instances by WTKit cells was very low despite the fact that the cells could be maintained indefinitely in liquid culture. Additionally in a separate colony assay, WTKit-infected cells formed greater numbers of colonies per 3,000 cells, when plated at a higher density (10,000 cells/dish) compared to those plated at 3,000 cells/dish. Together the results show that WTKit-expressing cells in the presence of SCF, showed proliferative ability in culture (Figure 6.3), but extremely

low clonogenic potential (Figure 6.4) which could be increased by plating at a higher cell density. Therefore this suggests that other factors (besides SCF) possibly produced in an autocrine or paracrine manner, were able to enhance the proliferation of WTKit-infected cells.

The relative number of colonies formed by the adherent and non-adherent fractions (Figure 6.4 A-B)was similar for each of the three populations but in all cases, the adherent cells formed fewer colonies than the non-adherent cells; this will be discussed futher below.

6.4.2 Colony morphology

Visual inspection revealed a marked variation in the morphology of the colonies formed by the different cell lines. The relative percentages of the different types of colonies formed by the cell lines are shown in Table 6.1 and representative examples have been shown previously in Figure 3.7. Using the criteria previously applied to the analysis of differentiation of myeloid leukemias (as discussed in chapter 3), the 'compact' colonies are suggestive of immature highly proliferative cells, the 'diffuse' of more differentiated cells types like mature macrophages and the 'mixed' a mixture of both, mature and immature cells (see figure 3.7). The non-adherent parental cells formed mainly compact colonies. However, the adherent cells in comparison formed higher proportions of diffuse or mixed colonies. This (along with the low total number of colonies - see Figure 6.4B) indicated that the adherent cell population were more differentiated or more readily underwent differentiation. WTKit adherent cells formed very few total colonies (Figure 6.4B) and the majority were of a mixed phenotype (Table 6.1), together indicating either that most of these cells were differentiated or that they required other factors to proliferate. The majority of non-adherent V816Kit cells formed compact colonies suggesting that the clonogenic cells

in the population were immature and highly proliferative, and they were not influenced by the presence of GM-CSF or SCF. The adherent V816Kit cells also formed mostly compact colonies with some variations with the addition of GM-CSF or SCF. Another type of colony formed by Kit expressing cells besides those discussed above, was termed a 'halo' colony which was essentially a 'mixed' colony with a compact ring of cells surrrounding it as shown in Figure 6.5 (A). This ring of cells was found to extend further out from the colony centre with time after plating in the presence of growth factor, suggesting that these cells could have migratory potential, a known feature of mature macrophages.

6.5 MORPHOLOGICAL AND PHENOTYPIC CHARACTERISTION

As shown in Figure 6.5(B) and 6.5(C) 'giant' cells were observed when V816Kit cells formed colonies in methylcellulose and when maintained in liquid culture respectively. However, it should be noted that the examples in Figure 6.5(B) illustrate the most extreme difference that was observed between 'normal' and 'large' sized cells. A wide range of cell sizes was observed in the Kit expressing MTHC cell populations. The relative number of the larger cells compared to the smaller cells was low in liquid culture. Similarly the proportion of colonies formed in methylcellulose containing these larger cells was relatively low. The majority of the large to giant cells in liquid culture were adherent, and an increase in the proportion of adherent cells was observed as the cultures became more dense. With prolonged culture of the adherent population, the larger cells were found to increase in size and granularity, with many displaying large vacuoles often encompassing smaller cells or cell debris.

6.5.1 Cell Phenotype

Cytocentrifuge smears of the different cell populations were stained with

Wright-Giemsa to show the cellular morphology (see section 2.2.2). The photographs shown in Figure 6.6 are representative of the proportions of the various phenotypes of cells in the total population. The parental MTHC were mainly non-adherent (on average 88%) and were of an immature myeloid phenotype (Figure 6.6a), while the small adherent fraction consisted of differentiated macrophages and neutrophils (Figure 6.6d). This was also the case with parental MTHC infected with the RUFneo vector alone. The non-adherent WTKit cells (Figure 6.6b) were heterogeneous with the majority of cells being similar to the parent type; most of the others were smaller in size and resembled mature neutrophils at various stages of differentiation. A few large cells displayed features of mature macrophages showing lighter stained areas in the cytoplasm. The adherent population (10-33%) of WTKit cells contained a higher proportion of these large cells as well as some small neutrophilic cells (Figure 6.6e). The non-adherent factor-independent V816Kit population was largely homogeneous and closely resembled the parental MTHC (see Figure 6.6c), but contained the occasional large cell with very obvious internal vacuoles which sometimes contained other cells (Figure 6.5C). There was an increase in the number of large to 'giant' cells with other cells bound to them in the adherent fraction of V816Kit cells (Figure 6.6f). The proportions of non-adherent and adherent V816Kit cells in culture varied dramatically with changes in the cell density, with a marked increase in adherent cells observed at high density.

6.5.2 Lineage characterisation by esterase staining

To further characterise the cell types observed (Figure 6.6) and to determine the differentiation state of the cells, the expression of lineage-specific esterases was analysed (as described in section 2.2.3) and the results are shown in Figure 6.7. The fields of view shown were chosen to illustrate most of the different cell types that

were observed upon enzyme staining and hence are not representative of the proportions of the various phenotypes of cells in the total population. The α-naphthyl acetate esterase is normally expressed by mature macrophages and the naphthol-AS-D-chloroacetate esterase by mature neutrophils (Yam *et al.*, 1971) . The non-adherent parental MTHC did not express either esterase, consistent with their immature state. The strong staining of the esterases by some of the Kit expressing cells indicated that, compared to the parental MTHC, they were more mature and the cell populations were more heterogeneous. The large to giant cells seen in the adherent fractions were macrophage-like cells but contained various levels of naphthol-AS-D-chloroacetate esterase. Enzyme staining separately for the two esterases showed that some large cells expressed high levels of both esterases and this double staining is probably the cause of the black colouring observed (Figure 6.7f) when the stains were applied consecutively. It was not clear if these cells were "bi-lineage" cells or if they were macrophages that had acquired the neutrophil esterase by phagocytosing other (neutrophilic) cells. The presence of these large cells in the V816Kit population as well as in the WTKit population maintained in SCF, indicated that signalling through Kit resulted in maturation of proliferating myeloid cells.

6.5.3 Phagocytic capacity

The ability of the adherent cells to phagocytose fluorescent beads was analysed as described in section 2.9.3. The results for the different cell populations are shown in Figure 6.8. The parental MTHC showed minimal phagocytic ability except for a few spontaneously differentiated mature macrophages. The WTKit cells again were extremely heterogeneous and ranged from no phagocytic ability to very high, with no obvious correlation between cell size and phagocytic capacity. Only the giant

cells in the V816Kit population remained adherent after washing and all showed phagocytic ability.

6.5.4 Cell surface marker analysis

Further characterisation of the MTHC by immunofluorescence as described in section 2.3.2 is shown in Figure 6.9. The "early" markers Thy1 and murine c-Kit, found on progenitor cells, were analysed as it was shown previously that the level of surface expression of these markers varied by MTHC varied with time in culture (see Chapter 3). The parental MTHC were maintained for 2 months in GM-CSF prior to the introduction of Kit and were shown to be heterogeneous in their expression of both markers (see Figure 3.5). The results in Figure 6.9 show that introduction of human Kit resulted in a decrease in expression of Thy1 and murine c-Kit compared to the parental MTHC, suggesting that the Kit-expressing populations were more mature. The WTKit cells also showed increased levels of mature markers of the granulocyte lineage (Gr1) and the monocytic/marophage lineage (Mac1), compared to the other cells, again indicating that they were more differentiated. The levels of these markers on V816Kit cells were low; however, it should be noted that the results shown in Figure 6.9 illustrate expression only by the non-adherent cells. The low numbers, variation in size of the adherent cells and fragility of the giant cells made it difficult to analyse this population using flow cytometry. The results that were obtained indicated that, in most cases, the profiles were similar to the non-adherent cells, except for sub-populations of adherent V816Kit cells that showed high levels of Gr1 and F4/80 antigen expression. This indicated that there was a greater difference between the non-adherent and adherent populations of the V816Kit cells when compared to the other MTHC, in agreement with the results shown in Figures 6.6 and 6.7.

6.6 TUMOURIGENIC POTENTIAL

Aliquots of $2x10^6$ non-adherent cells of either parental MTHC, WTKit MTHC or V816Kit MTHC, were injected sub-cutaneously into groups of 4 syngeneic (CBA) mice. The mice were monitored for the development of tumours for up to 3 months. Neither the parental MTHC nor the WTKit cells gave rise to tumours in any of the mice. In contrast, the V816Kit produced visible tumours after 2 weeks in 3 of the mice, with the 4th mouse in this group displaying a tumour at 6 weeks after injection. Analysis of this 'late-arising' tumour showed that it was partly intra-dermal and partly sub-cutaneous compared to the others that were sub-cutaneous. All tumours were excised and frozen or formalin fixed for phenotypic analysis, or dispersed to obtain tumour cells that were cultured *in vitro*. The tumour derived cells were able to proliferate in the absence of factor *in vitro* and were able to generate heterogeneous populations containing large adherent cells similar to those shown in Figure 6.6f. Sections obtained from paraffin-embedded tumour masses were stained with Haematoxylin and Eosin to examine the morphology of the cells, and are shown in Figure 6.10A. The cells show nuclear characteristics similar to malignant histiocytes, with many cells undergoing mitosis. The cells were found to invade surrounding fat (Figure 6.10B) and muscle tissue as well as blood vessels (Figure 6.10C). Sections of fresh-frozen tumour were stained for esterases. Variable staining was observed with some areas positive for expression of α-naphyl-esterase that contained a few strong staining larger cells (Figure 6.10D). Other areas showed positive staining for naphthol-AS-D-chloroacetate esterase expression (Figure 6.10E). This indicated that the tumours formed by V816Kit MTHC consisted of early and mature granulocyte and macrophage cells, thus resembing the cell populations that grew *in vitro*.

Figure 6.1: Colony formation by MTHC infected with retroviruses carrying human Kit encoding constructs.

The parent MTHC infected with retroviruses containing either RUFneo, c-kitRUFneo, or mutant kitRUFneo are represented as Vector, WTKit and V816Kit respectively. After 1 week of G418 selection in the presence of GM-CSF, cells were plated in triplicate (20,000 per dish) in methylcellulose containing either murine GM-CSF or no factor. Colonies containing more than 50 cells were scored 1 week after plating and the mean colony numbers +/- the SEM are shown with the * indicating an absence of colonies.

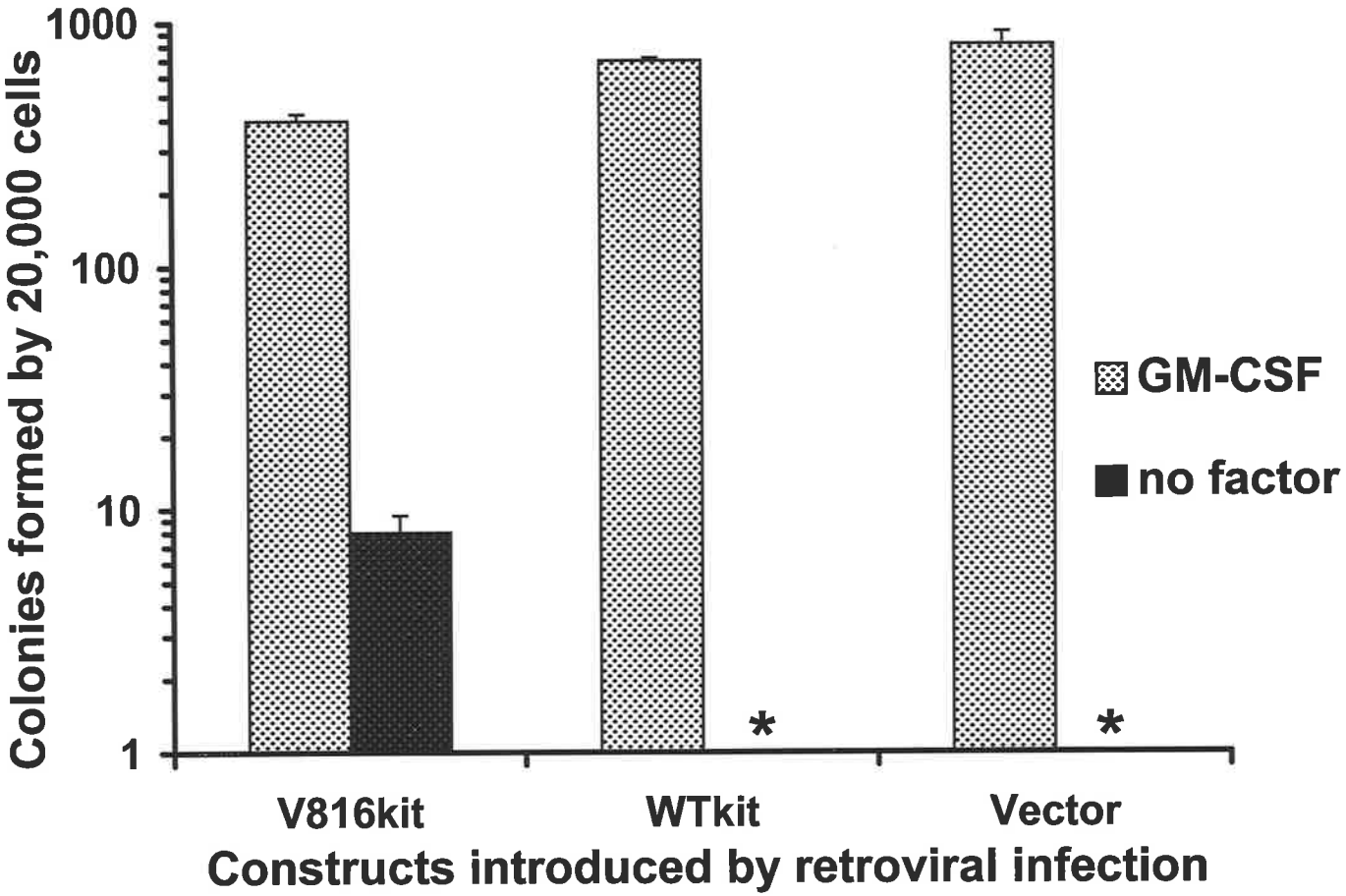
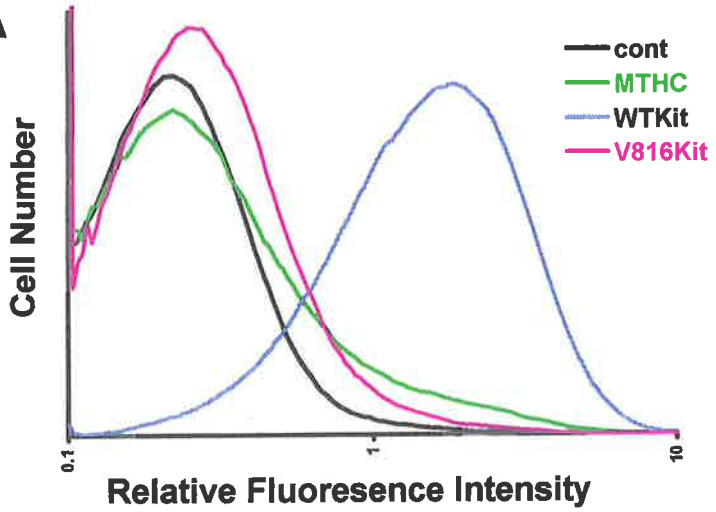

1000
100
10
1
Colonies formed by 20,000 cells
GM-CSF
no factor
*
*
V816kit
WTkit
Vector
Constructs introduced by retroviral infection

Figure 6.2 : Expression of human Kit as detected by A: Immunofluoresence and B: APAAP using mAb IDC3 (anti-human c-kit).

(A) The histogram shows the relative surface expression profiles of human Kit as detected with 1DC3 on parental MTHC cultured in murine GM-CSF, WTKit expressing MTHC maintained in human SCF and V816Kit expressing MTHC maintained without factor. The negative control corresponds to parental MTHC labelled without primary antibody and is representative of background.

(B) The red staining shown in the photographs indicates the presence of human Kit protein detected with 1DC3 by the APPAP method on the same populations of cells assessed by immunofluorescence in panel A: (1) parental MTHC, (2) WTKit expressing MTHC and (3) V816Kit expressing MTHC. The parental cells do not express human c-Kit and therefore represent a negative control. The bar in panel C represents 15 μm and provides the scale for panels 1-3

A


cont
MTHC
WTKit
V816Kit
Cell Number
0.1
1
10
Relative Fluoresence Intensity

B

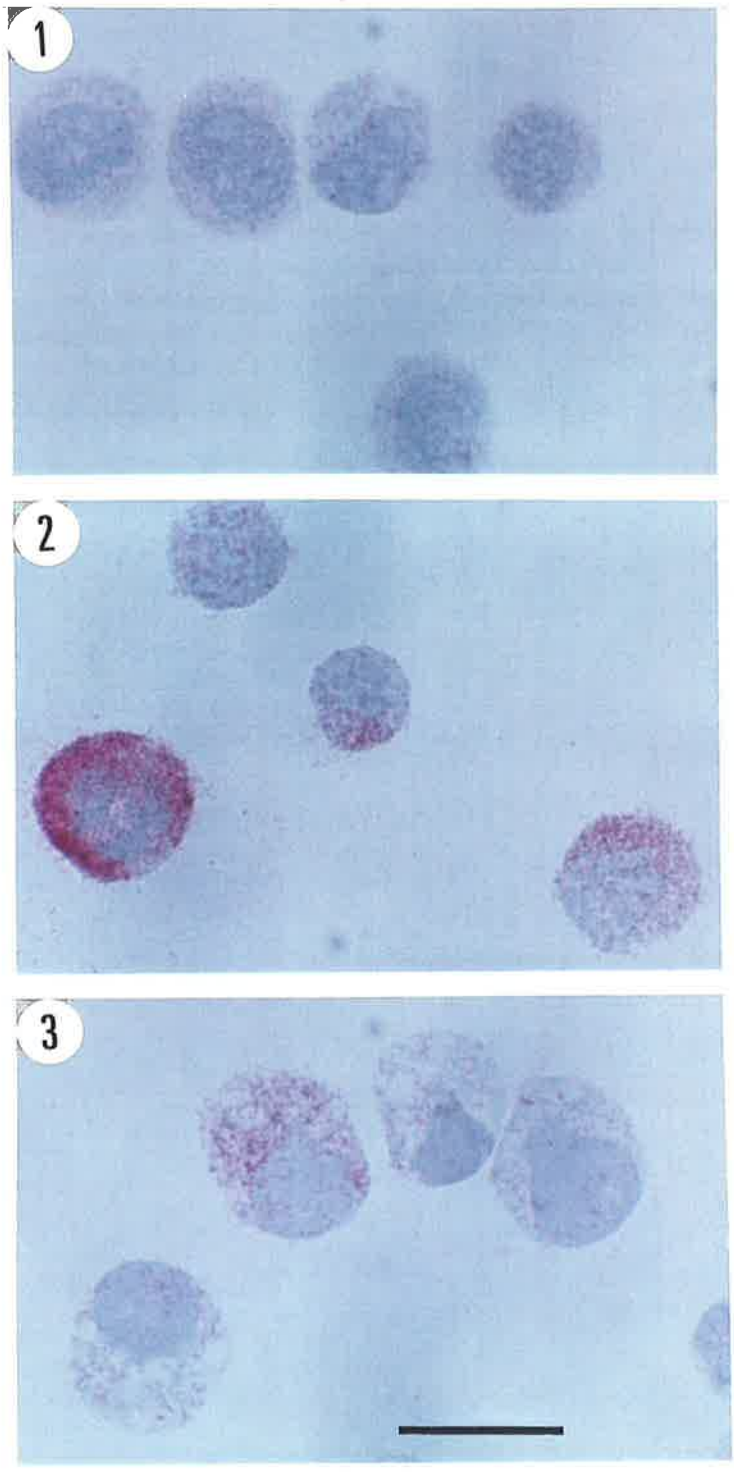

1
2
3

Figure 6.3: Growth of Kit expressing MTHC in liquid culture.

Parental MTHC maintained in murine GM-CSF, WTKit expressing MTHC maintained in human SCF and V816Kit expressing MTHC maintained without factor were assessed for proliferation as described in section 2.9.1(B). All cells were seeded in duplicate wells at 2,000 cells per 200µl.

The upper 3 panels show the number of viable cells recovered (mean +/- SD) at day 0, 2 and 4, after culture in medium supplemented with A: murine GM-CSF, B: human SCF and C: no added factor.

The middle 3 panels show the average number of cell divisions of the viable populations in the presence of D: 400U/ml murine GM-CSF, E: 100ng/ml human SCF and F: no added factor.

The bottom 3 panels show the survival of cells relative to the total viable cells at day 0 in culture containing G: murine GM-CSF, H: human SCF and I: no added factor.

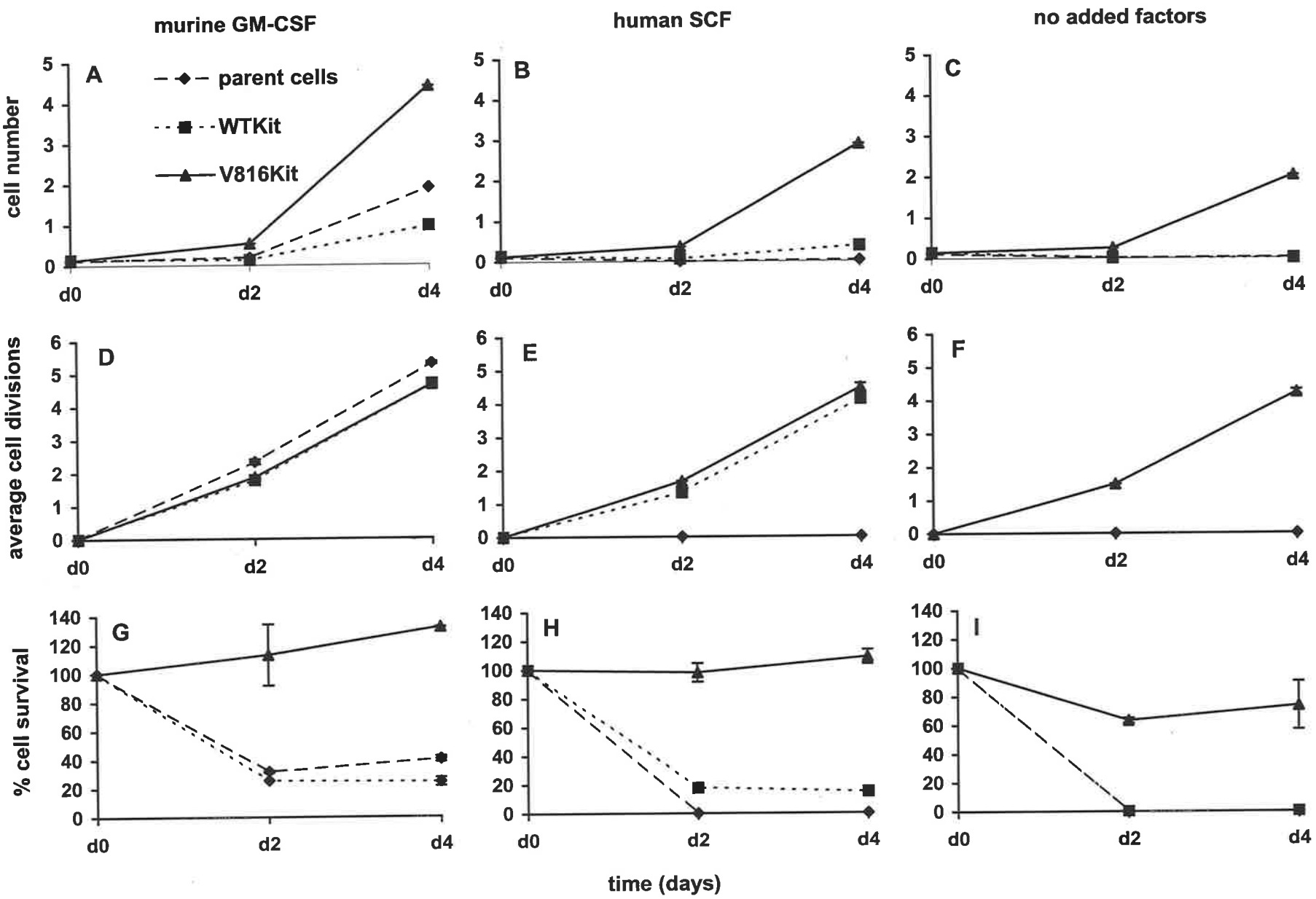

murine GM-CSF
human SCF
no added factors
A
B
C
D
E
F
G
H
I
parent cells
WTKit
V816Kit
cell number
average cell divisions
% cell survival
time (days)
d0
d2
d4

Figure 6.4: Clonogenic growth of Kit expressing MTHC.

Cells from the same cultures of parental, WTKit and V816Kit MTHC as for Figure 2, were plated at 3,000 per dish in methylcellulose containing either 400U/ml murine GM-CSF, 100ng/ml human SCF or no factor. The graphs show the average number of colonies +/- SEM at 2 weeks after plating.

Panel A shows the results for non-adherent cells collected from suspension and Panel B the results for adherent cells from the same culture, harvested by trypsinization.

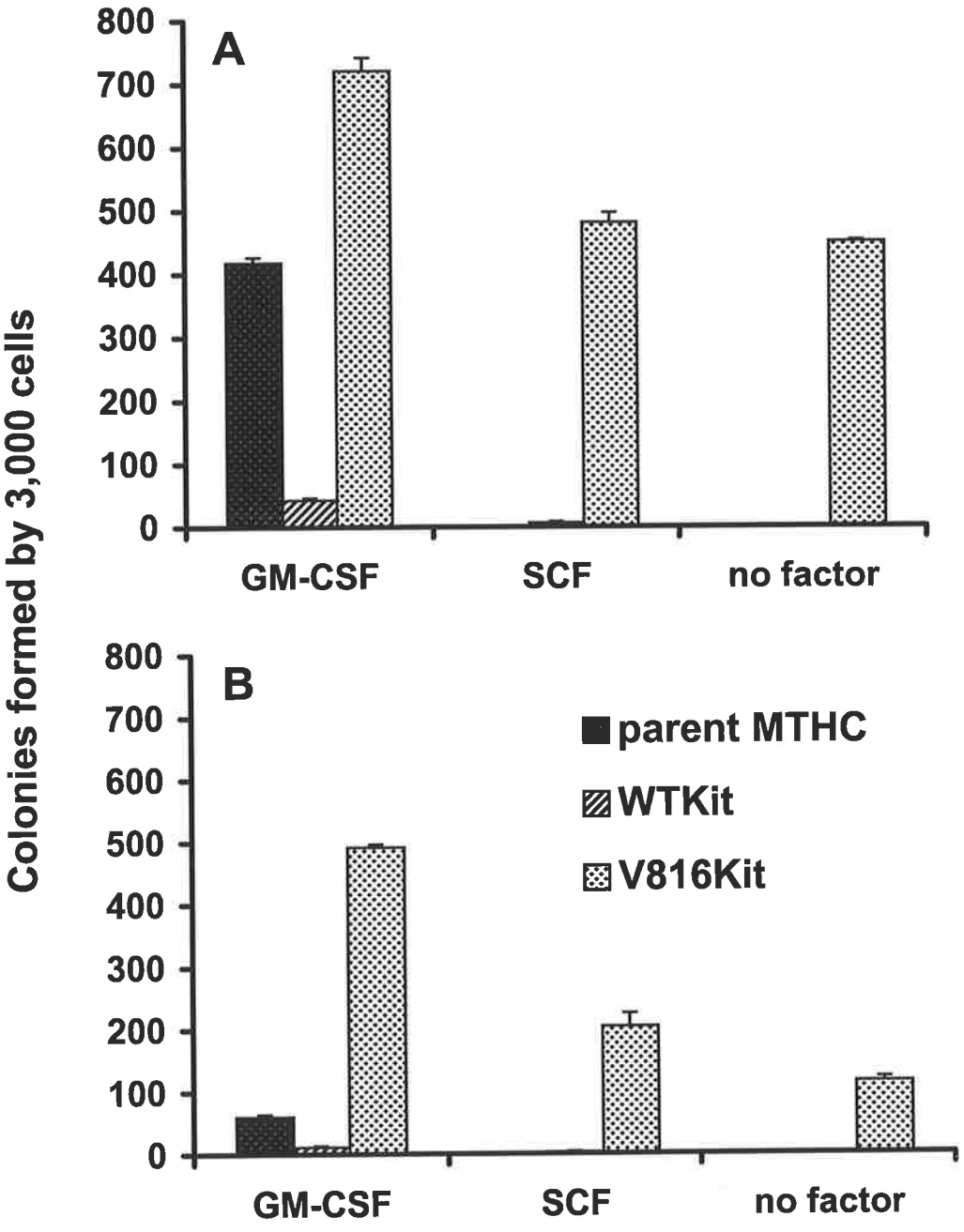

A
800
700
600
500
400
300
200
100
0
GM-CSF
SCF
no factor
B
parent MTHC
WTKit
V816Kit
Colonies formed by 3,000 cells

Table 6.1: Relative percentages of the various colony types formed by the MTHC cells.

The colonies formed in methylcellulose (Figure 6.4) were scored 3 weeks after plating as compact (an example was shown previously in Figure 3.7 A), diffuse (shown in Figure 3.7 B) and mixed (shown in Figure 3.7 C).

The numbers shown represent the percentage of the different colony types of the total number counted.

A	COLONY TYPE		
non-adherent cells	% COMPACT	% DIFFUSE	% MIXED
MTHC in GM	79	13	9
WTKit in GM	29	24	46
WTKit in SCF	45	38	17
V816Kit in GM	96	1	3
V816Kit in SCF	87	6	7
V816Kit without factor	88	3	9

B	COLONY TYPE		
adherent cells	% COMPACT	% DIFFUSE	% MIXED
MTHC in GM	23	27	50
WTKit in GM	8	17	75
WTKit in SCF	0	0	100
V816Kit in GM	81	5	14
V816Kit in SCF	65	9	26
V816Kit without factor	68	5	27

Figure 6.5: Morphology of the Kit-expressing MTHC cells and colonies.

Panel A shows an examples of a type of colony formed by Kit-expressing MTHC in methylcellulose. This colony is shown as a comparison to the colony types normally formed by MTHC (shown previously in Figure 3.7). This colony has been called a 'mixed halo' colony due to its characteristic compact centre with diffuse cells radiating outward surrounded by a compact ring of cells (halo).

(N.B. Due to the gradual migration of the 'halo' outward from the centre of these colonies with time, the number varied depending on the time after plating. Therefore all halo colonies were counted as mixed colonies - see Table 6.5)

Panels B and C show the presence of "Giant" cells in colonies and in liquid cultures, respectively, of Kit expressing MTHC.

Panel B shows the size comparison of the a 'giant cell' compact colony and a 'normal' compact MTHC colony.

Panel C shows the same comparison in liquid culture with the arrows indicating a non-adherent giant cell in the middle of the photograph and an adherent giant cell to the extreme right.

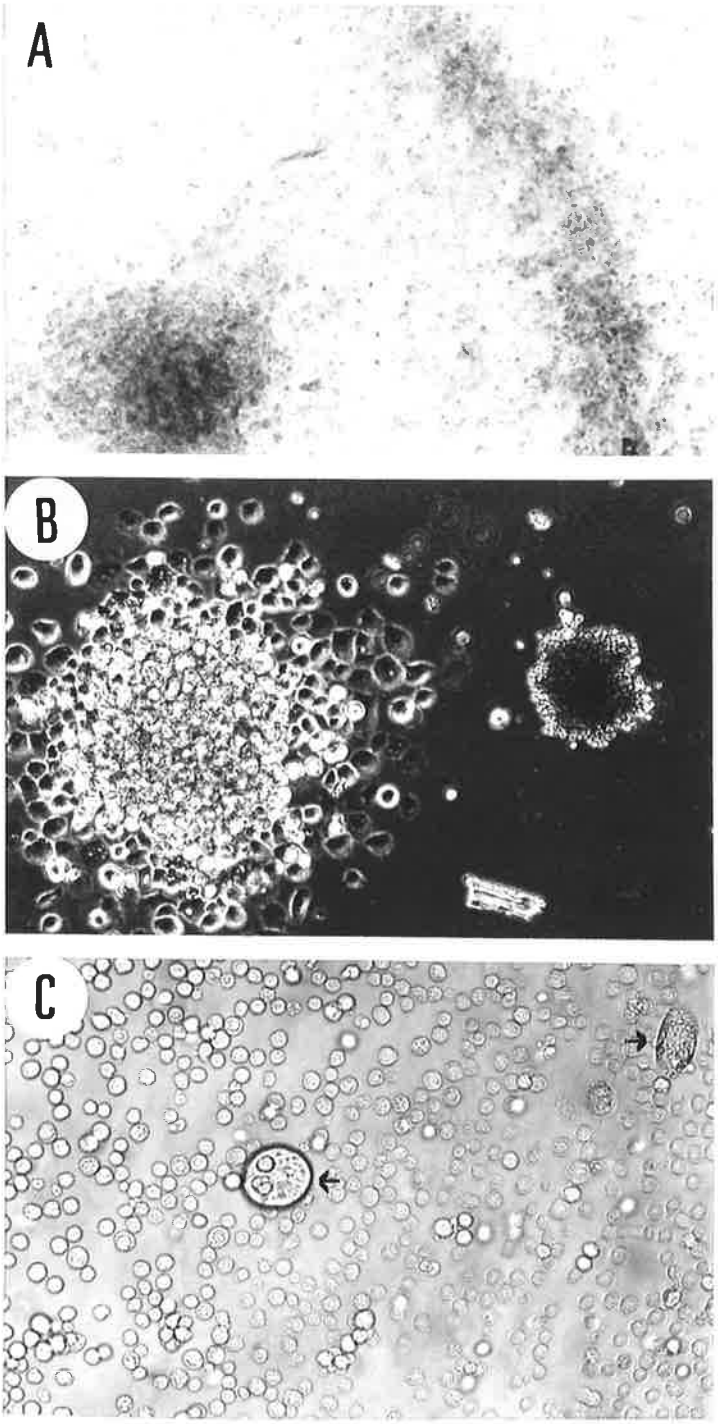

A
B
C

Figure 6.6: Morphology of non-adherent (a-c) and adherent (d-f) populations derived from liquid culture.

Cytocentrifuge preparations were stained with Wright-Giemsa. The fields of view shown are representative of the total cell populations. Panels (a) and (d) show non-adherent and adherent parent MTHC cultured in murine GM-CSF; panels (b) and (e)* show non-adherent and adherent WTKit expressing MTHC maintained in human SCF; panels (c) and (f) show non-adherent and adherent V816Kit expressing MTHC maintained without factor. The bar in panel (a) is equivalent to 38 μm and all photographs are at the same magnification.

* see additional notes

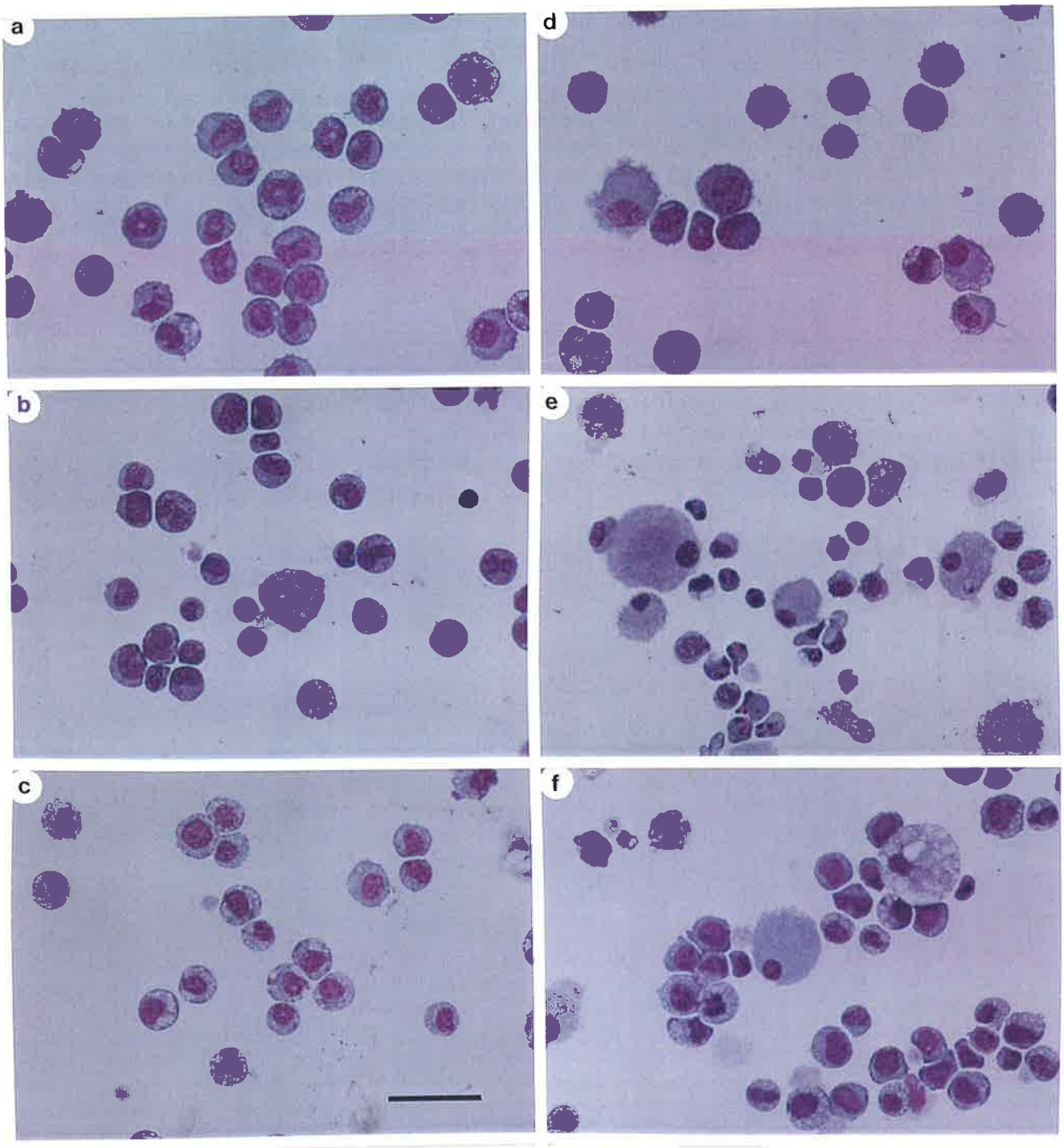

a
d
b
e
c
f

Figure 6.7: Esterase expression by the MTHC populations.

The cell groups are the same as those shown in Figure 6.6. Red staining is indicative of α-naphthyl acetate esterase and blue is indicative of naphtol-AS-D-chloroacetate esterase, with methyl green as the counterstain. Panels a and d show non-adherent and adherent parental MTHC cultured in murine GM-CSF, panels b and e* show non-adherent and adherent WTKit expressing MTHC maintained in human SCF and panels c and f show non-adherent and adherent V816Kit expressing MTHC maintained without factor. The bar in panel a is equivalent to 38μm and all photographs are at the same magnification.

* see additional notes

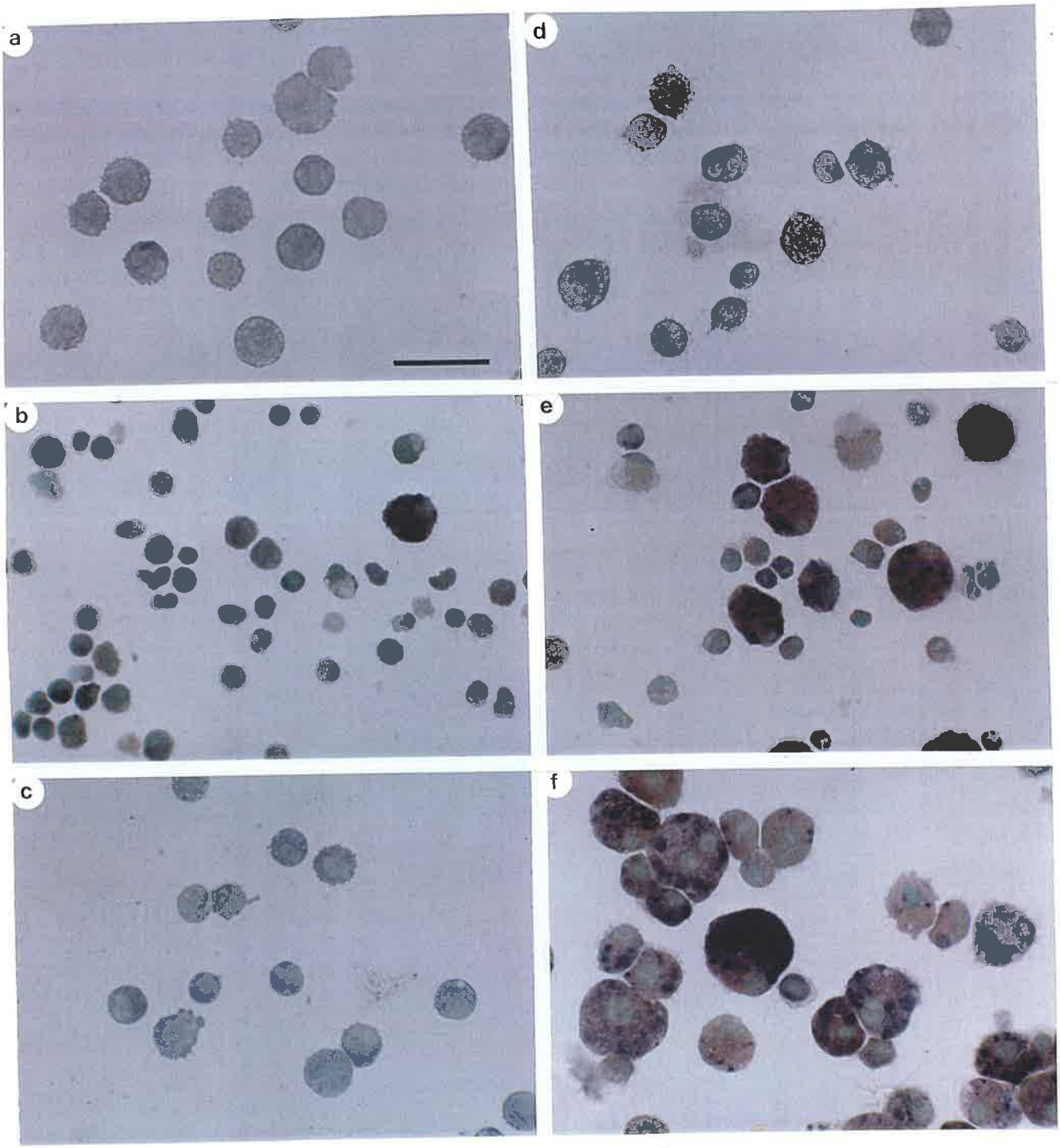

a
d
b
e
c
f

Figure 6.8: Phagocytosis of fluorescent beads by adherent MTHC.

The images in the two columns are of the same field of view as observed by phase-contrast microscopy shown on the left (A-C), and fluorescence microscopy shown on the right (D-F). The cells can be seen in the images on the left and the phagocytosed fluorescent beads on the right. The number of the cells in the field of view is indicative of the proportion of cells that were strongly adherent to glass in cultures of similar cell density.

Panels A and D show parental MTHC, panels B and E show WTKit expressing MTHC and panels C and F show V816Kit expressing MTHC. The cells were observed at an initial magnification of 40x.

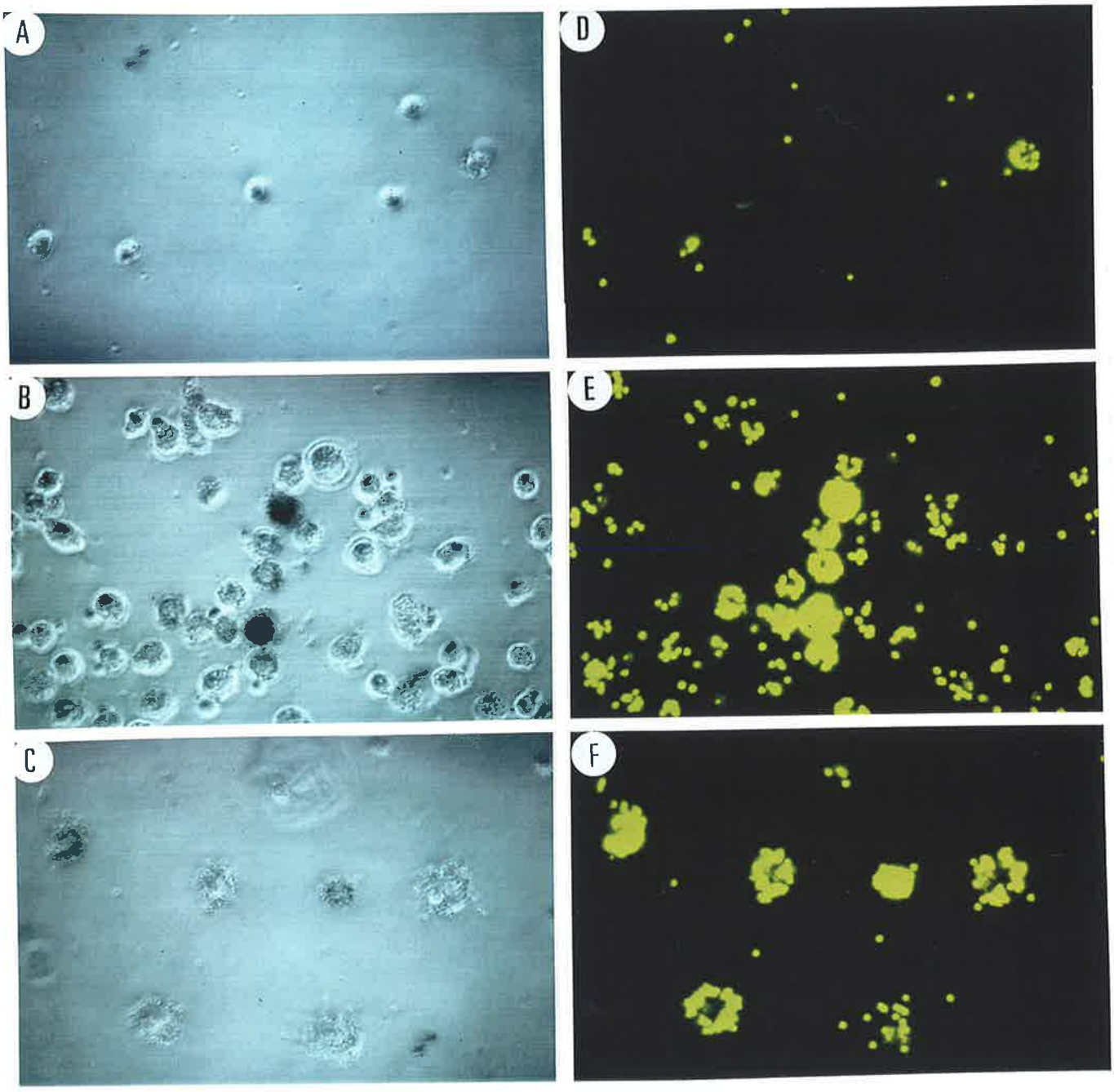

A
B
C
D
E
F

Figure 6.9: Analysis of cell surface marker expression by immunofluoresence.

The histograms show the relative surface expression profiles of the indicated cell surface antigens by non-adherent parental MTHC cultured in murine GM-CSF, WTKit expressing MTHC maintained in human SCF and V816Kit expressing MTHC maintained without factor. The cells were analysed by immunoflouorescence using mAbs ACK2 (anti-murine c-kit), 30H12 (anti-Thy1.2), MI/70 (anti-Mac1 biotin conjugated), 8C5 (anti-Gr1 FITC conjugated) or F4/80 (anti-macrophage marker). The primary antibodies were detected with either FITC-conjugated sheep anti-mouse immunoglobulin, FITC-conjugated sheep anti-rat Ig or FITC-conjugated Strepavidin (Amersham, U.K.). The negative control shown in each panel corresponds to parental MTHC labelled with the relevant secondary antibody used in each case, and is representative of background.

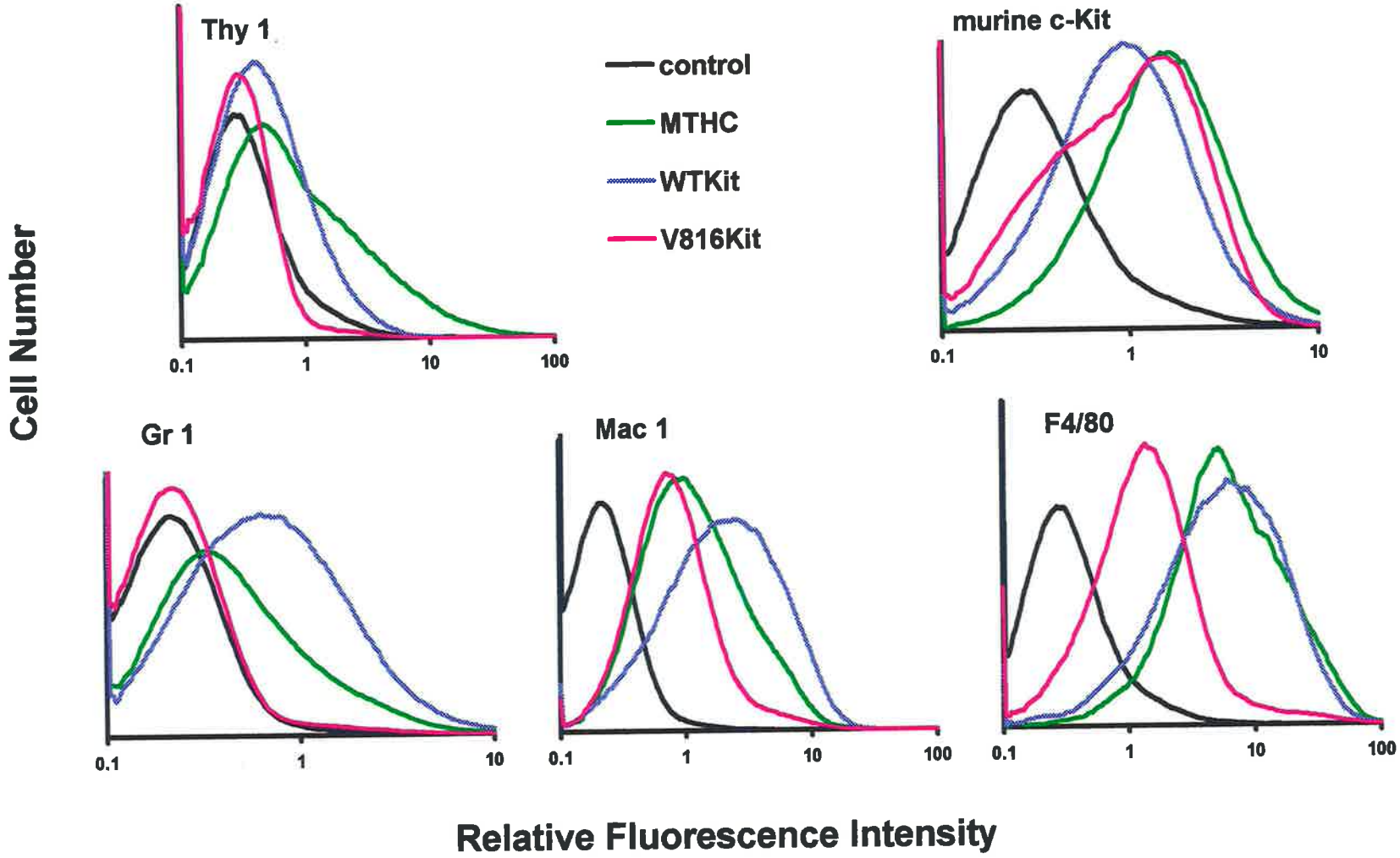
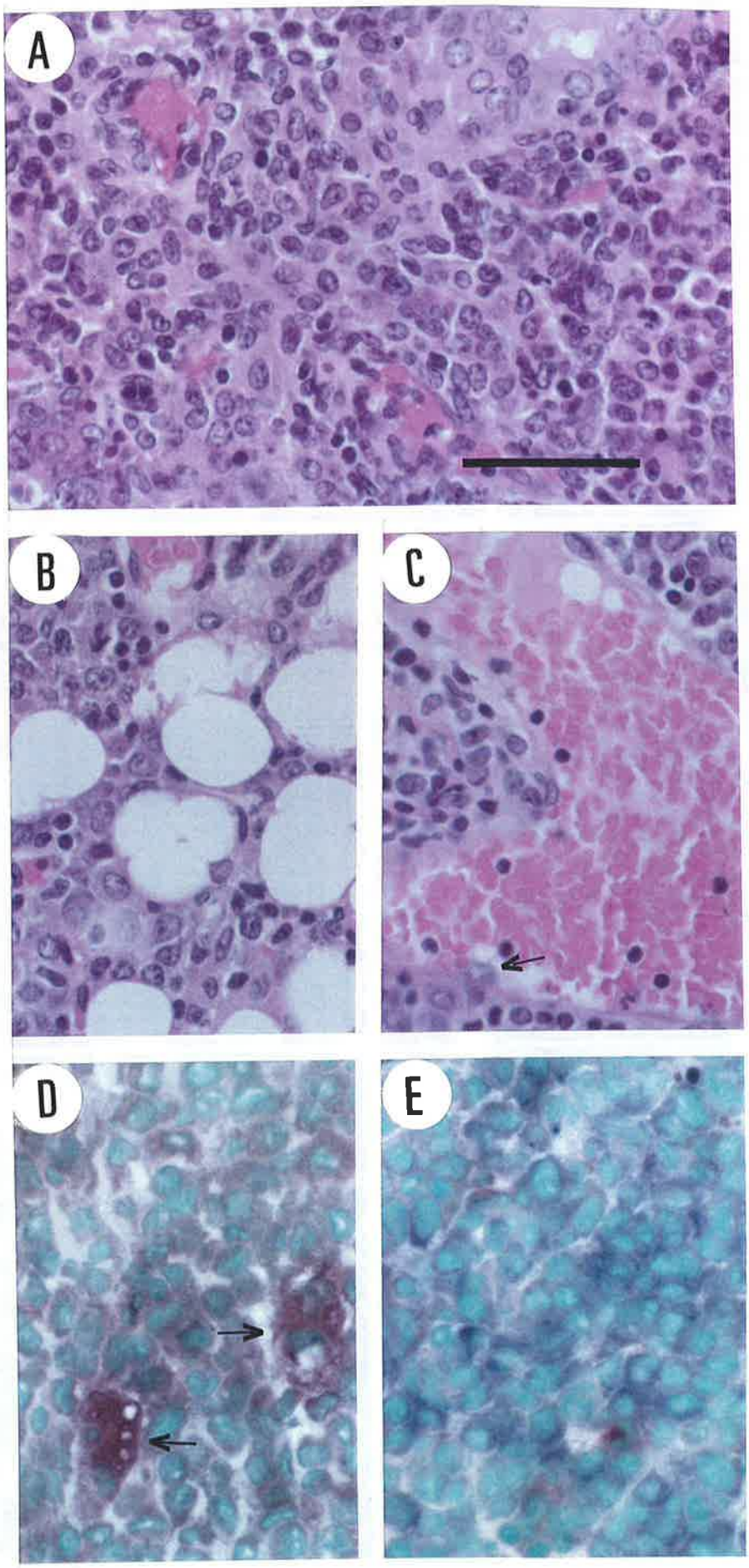

Thy 1
control
MTHC
WTKit
V816Kit
murine c-Kit
Gr 1
Mac 1
F4/80
0.1
1
10
100
Cell Number
Relative Fluorescence Intensity

Figure 6.10 : Micrographs showing the histopathology of tumours formed by V816Kit-expressing MTHC.

Panels A, B and C show Haematoxylin and Eosin staining (see 2.2.2) of a paraffin embedded tumour section (see 2.2.1) and D and E show esterase expression in frozen sections. Panel A shows a region through the centre of the vascularised tumour, B and C show invasion of the tumour cells into surrounding fat tissue and a blood vessel (marked with an arrow) respectively. Panels D and E show photographs of fresh frozen sections of the tumour (see 2.2.1) that were stained for the presence of lineage specific esterases (see 2.2.3). Small regions of staining for predominantly macrophage or neutrophil esterase are shown in panels D and E respectively. The arrows in photograph D indicate the larger cells showing phagocytosis of other cells. The bar in panel A represents 38 μm and provides the scale for panels A-E.


A
B
C
D
E

6.7 DISCUSSION

Continuous over-expression of human c-Kit (WT) or an activated Kit mutant (V816) in MTHC (which normally dependent on GM-CSF) allowed growth in the presence of human SCF alone, or in the absence of factor, respectively (see section 6.2). There were two potentially important observations: (1) a difference between the effects of Kit activity compared to GM-CSF activity in MTHC and (2) a difference between WTKit activated by SCF and the constitutively activated V816Kit mutant.

The level of V816Kit expression could not be accurately determined by immunofluorescence. However, the level of expression of murine c-Kit on MTHC (Figure 6.9) was similar or higher than the levels of introduced human WTKit (Figure 6.2A) on the same cells (as determined by a similar 'peak shift').Therefore, the results suggest that WTKit in MTHC was not 'over-expressed', and the effects observed were due to enforced expression of Kit in the presence of SCF.

Both WTKit plus SCF and V816Kit allowed proliferation of MTHC in the absence of GM-CSF. Apart from proliferation, Kit was also able to induce phenotypic changes in MTHC. Introduction of either WTKit (plus SCF) or V816Kit resulted in more heterogeneous populations of cells with an increase in the number of large to giant adherent cells. These cells were shown by several criteria to be mature differentiated cells of the GM lineage which possessed phagocytic activity. This differentiation and maturation phenomenon was only observed in a proportion of the cells, however clonal cell populations obtained from the different types of colonies in methylcellulose (see Table 6.1) all gave rise to heterogeneous cell populations in culture containing mature adherent cells. Therefore, the phenotypic heterogeneity in the cell populations in liquid culture and in colony assays was probably due to intrinsic properties that induced activation of differentiation pathways, and not a result

of heterogeneity in the initial cell population. These cells may provide a useful model for investigating differentiative signals induced by Kit activity in myeloid cells.

The other important observation was the difference between WTKit (plus SCF) and V816Kit activity in MTHC. WTKit (plus SCF) induced more differentiated cells with a decreased growth rate and lower clonogenic potential than parental MTHC, whereas V816Kit induced mixed populations of immature and mature cells with, increased survival, growth rates and clonogenic potential. This indicated that the mutant Kit was able to provide a maturation effect in a proportion of MTHC but this was not linked with decrease in proliferation of the majority of the population. A possible reason for this could lie in differences between the actions of WTKit (plus SCF) and V816Kit in MTHC. It was recently shown that expression of the corresponding murine mutant, D814YKit, in the mast cell line IC-2 resulted in degradation of the phosphatase PTP1C (Piao *et al.,* 1996), also known as HCP, SHP1 and SHPTP1, which is the product of the motheaten (*me*) gene. This phosphatase associates with activated Kit and is a negative regulator of Kit signalling (Yi and Ihle 1993; Paulson *et al.,* 1996) and IL-3 receptor (Yi *et al.,* 1993) signalling, and is also known to interact with Grb2, mSos1 and Vav (Kon-Kowslowski *et al.,* 1996). Degradation of this phosphatase is thus likely to occur in V816Kit expressing MTHC and would result in loss of negative regulation of many of PTP1C substrates. As many of the substrates, including Kit itself, are haemopoietic growth factor receptors, this would result in enhanced activity of all these proteins and may be a contributing factor to the high growth capacity observed in phenotypically mature V816Kit expressing MTHC populations. The murine form of this mutant was also found to exhibit altered sites of autophosphorylation and altered substrate specificity, (Piao *et al.,* 1996).

V816Kit is likely to behave similarly, which would further explain differences in the effects of this mutant compared to WTKit+SCF.

The expression of V816Kit in MTHC resulted in the generation of heterogeneous cell populations *in vitro* in the absence of factor, as well as in the production of tumours. In both cases the cells were at various stages of differentiation with some cells of giant size displaying phagocytosis of other smaller cells. The giant cells grown *in vitro* phenotypically resembled histiocytes due to their vacuolated cytoplasm and bi-nucleation. Additionally, the tumour cells showed histopathological features similar to the malignant cells in murine malignant histiocytosis induced by a transforming murine retrovirus carrying the Ha-*ras* oncogene (Eckman *et al.*, 1995). The only previous report of differentiation of MTHC was in response to TNF-α / IL-4 that resulted in the production of mature macrophages (Gonda *et al.*, 1993). In this chapter, the results showed V816Kit could induce a differentiative effect in MTHC, producing heterogeneous populations of myeloid (GM) cells at various stages of differentiation including histiocyte-like cells.

There have been two reports of patients showing evidence of histiocyte-like cells linked with leukemia. This has lead to a proposal for the classification of a new type of acute histiocytic leukemia - AML-M5c (Laurencet *et al.*, 1994) and as acute monocytic leukemia with histiocytic differentiation (Esteve *et al.*, 1995). These histiocyte-like cells contain large often kidney shaped nuclei with broad irregular membranes, granular chromatin and prominent nucleoli - features similar to the nuclei of the tumour cells shown in Figure 6.10. It has been proposed that these diseases are caused by the proliferation of activated macrophages / histiocytes (Laurencet *et al.*, 1994), a phenomenon we have observed in clonogenic assays of V816Kit MTHC (Figure 6.5(B)). Therefore the histiocyte-like giant cells we have observed *in vitro* and

in vivo that were derived from cell populations expressing oncogenic Myb and oncogenic Kit may be of particular relevance in investigating these types of leukemia, and will be discussed further in chapter 7.

Chapter 7: DISCUSSION

The proto-oncogenes c-*myb* and c-*kit* both play a critical role in normal haemopoiesis and, like other proto-oncogenes, de-regulation of normal expression and/or mutations causing structural alterations to their products can result in oncogenic activation. Both *myb* and *kit* genes have been implicated in human and murine malignancies (see sections 1.3.3 and 1.5.2). The aims of the study presented here were to investigate the effects of oncogenic forms of Myb and Kit individually and co-operatively in primary haemopoietic cells using *in vitro* and *in vivo* experimental systems.

7.1 CONCLUSIONS

The results shown in Chapters 3-6 have provided further insight into the function and activity of these two oncoproteins in murine haemopoietic cells.

7.1.1 Structural alteration of Myb is not required for transformation

In murine and avian haemopoietic system, over-epression of truncated forms of Myb result in oncogenic activation and the ability to cause transformation of cells *in vitro* and lead to leukaemias *in vivo* (see section 1.3). However in humans, structural alterations have not been reported in c-Myb, although amplification of an otherwise normal human *c-myb* gene has been detected in acute myelogenous leukaemic cell lines (Pelicci *et al.,* 1984; Barletta *et al.,* 1987). The results discussed in chapter 3 (Ferrao et al 1995) showed that in the murine system, structural alteration of Myb was not essential for transformation of immature myeloid cells *in vitro*, however enforced expression of structurally altered (truncated) Myb resulted in a higher level of transformation (as assessed by clonogenic potential) and a more complete block in differentiation, when compared to full length Myb. Therefore, it is

possible that abnormal expression of Myb could be a causative factor in human myeloid diseases.

7.1.2 Activation of Myb - an early step ?

As discussed in the previous chapters, supply of an exogenous growth factor is necessary for the survival of MTHC cells *in vitro*, and acquistion of CSF-independence is a critical step for tumourigenicity (Gonda et al 1989a). Growth factor independence is also an important step for induction of myeloid leukaemias caused by avian retroviruses (Metz et al 1991) and has been shown to be essential in many studies for 'complete' transformation of haemopoietic cells of the murine and avian systems (reviewed in Gonda 1991). In these studies the most consistent observation is that expression of activated forms of Myb can contribute to, but is insufficient for the development of a malignant phenotype, suggesting that subsequent mutations in co-operative oncogenes are required. The results discussed in chapter 4 seem to correspond with this notion, since the presence of activated Myb in haemopoietic cells of mice did not induce any detectable haemopoietic disorder.

Since it is widely accepted that cancer is a multi-step process, for the pupose of this investigation it was important to look at the effects of other oncogenes that were able to co-operate in causing 'complete' transformation of cells. Expression of GM-CSF (Gonda et al 1989a) and of activated forms of the GM-CSF β chain receptor (McCormack and Gonda - unpublished), have been shown to result in factor-independence of MTHC, which is associated with a gain in tumourigenic potential (Gonda et al 1989a; Gonda 1991).

7.1.3 Myb and Kit can co-operate in transformation

Interest in Kit as a potential co-operative factor began with evidence that activating mutant forms could confer factor-independence in myelomonocytic FDC-

P1 cells (Kitayama et al 1995), normally requiring GM-CSF (or IL-3) for survival similar to early myeloid MTHC. The results in chapter 5 and 6 showed that continuously expressed mutant Kit could act co-operatively with activated Myb to cause 'complete' transformation of murine myeloid cells. Activated Kit caused an increase in the survival and differentiation of MTHC and provided tumourigenic potential (chapter 6).

7.1.4 Kit: a role in differentiation of megakaryoctic and mast cell lineages

The results presented in chapter 5 also showed that enforced expression of activated Kit alone could increase the production of megakaryocytes and mature mast cells from primary haemopoietic cells, suggesting that activated forms of Kit could be linked with malignancies of these two lineages. c-Kit is normally expressed on human megakaryocytes (Avraham et al 1992a) and, unlike other mature cells of haemopoietic origin, human mast cells also express high levels of c-Kit (Mayrhofer et al 1987). c-Kit is critical for mast cell development (reviewed in Galli et al 1994), and megakaryocytes as well as mast cells have been shown to respond to SCF (Avraham et al 1992a,b; Bischoff and Dahinden, 1991). Furthermore, the expression of c-Kit and growth response to SCF was found to be a common feature of human megakaryoblastic cell lines (Hassan and Freund, 1995).

In terms of activated mutant Kit, most of the recent research has focussed on mast cell malignancies. In a study on mastocytosis patients (Nagata et al 1995), bone marrow biopsies from all four patients with the V816Kit mutation, showed diffuse mast cell lesions and increased numbers of megakaryoctes that were abnormal in appearence (as discussed in chapter 5). This mutation was also identified in human (Furitsu et al 1993; Nagata et al 1995) and rodent (Tsujimura et al 1994; Tsujimura et al 1995) malignant mast cells. Other mutations in the juxtamembrane domain of the

intracellular portion of Kit have also been associated with neoplasias of the mast cell lineage. A deletion of 21bp encoding seven amino acids 573-579 in murine mastocytoma FMA3 cells (Tsujimura et al 1996) and a point mutation resulting in an amino acid substitutution of Gly for Val at amino acid 559 (G559Kit) in human HMC-1 cells (Furitsu et al 1993; Kitayama et al 1995), have both been shown to confer factor-independent dimerization, leading to constitutive activation. Expression of the murine mutant forms of Kit corresponding to human V816Kit and G559Kit in BM gave rise to mast cell colonies as well as 'mixed' colonies containing megakaryocytic cells (Kitayama et al 1996). Taken together, these data associate Kit with the mast cell and megakaryocytic lineages, and strongly implicate activated mutant forms of Kit with mast cell malignancies.

7.1.5 Kit: a link with myeloid malignancies

In vitro colony assays showed that introduction of the mutant form of murine Kit corresponding to human V816Kit into bone marrow cells gave rise to factor independent CFU-GM that were increased in number by the addition of SCF (Kitayama et al 1996). Additionally, two of four patients with mastocytosis, displaying the V816Kit mutation (as discussed above), also showed dysplastic features of the myeloid lineage (Nagata et al 1995). Other mutations in Kit have also been reported in cases of myeloproliferative disorders. A point mutation in the extracellular region of Kit leading to an amino acid substitution of Asp to Asn at codon 52, was detected in two patients with primary myelofibrosis and one patient with chronic myelogenous leukaemia (Nakata et al 1995; Kimura et al 1997), but the activity of this mutant form of Kit is unknown. In another study, c-Kit was found to be phosphorylated in the absence of exogenous SCF in a number of patients with acute myeloblastic leukemia, although the presence of mutations in Kit was not

determined (Ikeda et al 1991). Taken together, all these reports suggest a link between Kit mutations and disorders of the myeloid lineage. However, in cases of AML, only one patient sample (AML-M2) of 33 cases examined was positive for the presence of the V816 mutation in Kit (SR Cole and LK Ashman, unpublished data). The results shown in chapter 6, suggest an explanation for this finding, in that V816Kit provided a strong differentiative, as well as proliferative and survival stimulus for early myeloid cells. Thus, similar mutant forms of Kit may be associated with myeloid malignancies of differentiated cell types.

It is important to emphasise that all the effects of V816Kit were not identical to that of WTKit plus SCF possibly due to altered substrate specificity of the mutant Kit and/or its ability to result in degradation of HCP (as discussed in chapter 6). Hence, other mutant forms of Kit or c-Kit activated by autocrine production of SCF, could bring about haemopoietic malignancies of different phenotypes.

Expression of WTKit (in the presence of SCF) was able to sustain growth of factor-dependent early myeloid cells and induce differentiation (chapter 6). SCF is an important growth and differentiation factor for normal early hemopoietic cells (reviewed in Galli et al 1994). Expression of c-Kit is detected in AML cells (see section 1.4.3 and 1.5.2) and studies have shown that SCF can induce a proliferative response in AML blast cells (see section 1.5.2). However results from one study showed that in AML, c-Kit protein was rarely over-expressed and gene amplification or autrocrine production of SCF was not observed (Cole et al 1996). In view of the results shown in chapter 6, it is possible that activation of Kit by over-expression and/or autocrine SCF production could provide differentiative effects in malignant cells and therefore be associated with leukaemias of mature cell lineages.

7.1.6 Myb and Kit in histiocytic malignancies?

The results discussed in chapter 6 showed that both WTKit in the presence of SCF and V816Kit could lead to the production of differentiated histiocyte-like cells when expressed in early myeloid MTHC. This is paricularly relevant to two cases of patients with acute histiocytic leukemia - AML-M5c (Laurencet et al 1994) and acute monocytic leukemia with histiocytic differentiation (Esteve et al 1995). The heterogeneous cell populations observed in these patients resemble those shown and discussed in chapter 6 (myeloid cells at various stages of differentiation including mature macrophages, neutrophils and large histiocyte-like cells). Additionally, it has been proposed that these diseases are caused by the proliferation of activated macrophages / histiocytes (Laurencet et al 1994), a phenomenon also observed and discussed in chapter 6 (see Figure 6.5B). This provides a link between the oncogenes Myb and Kit and these specific leukaemias.

7.2 FUTURE RESEARCH

Due to the reasons discussed above and in chapter 6, it will be important to test malignant cells from pateients with AML-M5c for mutations in c-*kit* and c-*myb*. The expression levels of these and other closely related proto-oncogenes would also be of interest in these particular leukaemias. It would also be important to analyse other types of leukaemias involving differentiated cell types (especially megakaryoctic, monocytic and granulocytic) for constitutively activating mutations in Kit, over-expression of the c-Kit protein or autocrine production of SCF.

The co-operative effects of activated Myb and activated Kit were investigated (Chapter 6) by introducing Kit into cells previously immortalized by Myb and maintained in GM-CSF. These MTHC were all of the GM lineage prior to the

introduction of Kit, thereby restricting the analysis of the effects of Kit to cells committed to these lineages. *In vitro* studies using MTHC cultured in other growth factors such as IL-3 and Epo or Tpo, prior to the introduction of Kit, could facilitate the production of other cell lineages (mast cell and megakaryocytic) that Kit is normally expressed in. However, co-infection of primary FLC with activated Myb and activated Kit, after three weeks in the absence of added growth factor, resulted in the production of myeloid cells, mostly mature macrophages or neutrophils (chapter 5). Therefore, the *in vitro* experiments seem to suggest that Myb 'selects' for cells of the GM lineages by specifically inducing their immortalization - maintaining them in an early proliferative state, and providing a growth advantage while cells of the other lineage spontaneously differentiate. If the cell lineages obtained were determined by the specificity of Myb, it is possible that Kit could be involved in other types of malignancies depending on co-operative factors. Other *in vitro* studies investigating the effects of Kit in factor-dependent cell lines immortalized by other oncogenes besides Myb would be of interest.

Due to time restrictions, a bone marrow reconstitution experiment analysing the effects of activated Myb and Kit together, was not included in the investigations presented here. However, it is important to determine the phenotype of recipient mice reconstituted with bone marrow co-infected with CT3Myb and V816Kit. This experiment could provide further links between the co-operative effects of these two oncogenes and specific malignancies. Reconstitution of mice with bone marrow infected with the murine form of V816Kit alone resulted in B cell lymphomas in a few cases (Kitayama et al 1996). This result is unexpected since most experiments link Kit with other lineages (see above), and may be due to the specificity of the promoter used for expression or may even reflect a variation in latencies of

malignancies of different lineages. A similar experiment with a mutant form of a closely related RTK, v-*fms* (see figure 1.3A) resulted in abnormalities of multiple haemopoietic lineages (Heard et al 1987). Therefore, from the results shown here and in other studies as discussed, a bone marrow reconstitution experiment analysing the effects of V816Kit alone or together with CT3Myb would be also be of importance.

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APPENDIX

A1.1 Reagents for Immunofluoresence, Immunocytochemistry and Immunohistochemistry.

Esterase fixative: 100mg Na_2HPO_4, 500mg KH_2PO_4, 225ml acetone and 125ml formalin (40% formaldehyde in water) were added to 150ml distilled water. The solution was stored at 4^0C

Pararosanalin: 0.2g pararosanalin hydrochloride (Sigma) was added to 10ml of 20% v/v HCl in water and incubated at 56^0C for 30 minutes in the dark. The solution was stored at RT in the dark.

Phosphate buffer pH 6.3: 3.5g KH_2PO_4 and 1.1g Na_2HPO_4 was added to 500ml of distilled water gradually while stirring. The solution was stored at 4^0C.

'Non-specific' esterase substrate solution: 0.3ml of pararosanalin solution was mixed with 0.3ml of 4% sodium nitrite solution and allowed to stand for 1 min. This was added to a freshly made solution of 30 ml 0.066M phosphate buffer pH 6.3 containing 1ml of 10mg/ml α-naphthyl acetate (Sigma) in acetone. The final pH was adjusted to 6.1 with 5M NaOH.

Phosphate buffer pH 7.4: 0.87g KH_2PO_4 and 3.84g Na_2HPO_4 was added to 500ml of distilled water gradually while stirring. The solution was stored at 4^0C.

'Chloroacetate' esterase substrate solution: 5mg napthol-AS-D-chloroacetate (Sigma) was diluted in 2.5 ml N-N dimethylformamide and then added to a fresh solution of 38ml phosphate buffer pH7.4 containing 20mg Fast Blue (Sigma).

2% methyl green counterstain: 1g methylgreen (Sigma) was added to 50ml distilled water ans shaken till dissolved. Contaminating metylviolet was removed by extraction with 50ml chloroform, repeated twice.

Glycerol/Glycine mountant: 1.4 g glycine was added to 100ml H_2O and the pH adjusted to 8.6 with NaOH. 30ml of this solution was added to 70ml glycerol and stored at RT.

Phosphate buffer pH 6 (0.1 M): 6ml of 1M Na_2HPO_4 and 44ml 1M NaH_2PO_4 were mixed and the volume made up to 500ml with distilled water.

PBS: 8g NaCl, 0.2g KCl, 1.65g Na_2HPO_4 and 0.2g KH_2PO_4 per litre of distilled water, adjusted to pH 7.4 with NaOH or HCl if necessary.

TBS: A solution of 1.5M NaCl was buffered to a pH of 7.6 with 0.5M Tris-HCl.

Gill's Haematoxylin: In the following order was added together; 730 ml distilled water, 250 ml ethylene glycol, 2 g Haematoxylin (anhydrous), 0.2 g sodium periodate, 17.6 g alumininum sulphate ($Al_2(SO_4)_3.18H_2O$) and 20 ml glacial acetic acid. The solution was stirred for 1 hour at RT. Each batch was allowed to mature for 4 weeks prior to use.

Scott's gentle alkaline solution: 3.5g $NaHCO_3$ and 20mg $MgSO_4.7H_2O$ were dissolved in 1L of distilled water.

A1.2 Reagents for DNA techniques.

100 x TE buffer: 12.1 g Tris was dissolved in 50 ml H_2O and the pH adjusted to 7.5 with HCl. 3.72 g EDTA was added and the volume made up to 100ml with H_2O. This solution was diluted as required.

10 x CIP Buffer: A solution of 10 mM $ZnCl_2$, 10 mM $MgCl_2$ and 100 mM Tris-HCl pH 8.3. This buffer remains as a precipitate and requires vortexing prior to use.

50x TAE: 100ml 0.5M EDTA pH 8 was mixed with 57.1ml glacial acetic acid and made up to 1L with H2O, to which 242 g Tris was added and dissolved. This stock solution was diluted as required.

A1.3 Reagents for bacterial culture.

LB medium: 5 g Bacto-yeast extract (Difco, USA, Cat. No. 012-01-7), 10 g Bacto-tryptone (Difco, USA, Cat. No. 0127-01-7) and 10 g NaCl were dissolved in 1L of H_2O and the pH adjusted to 7.4 with 1M NaOH. This solution was sterilized by autoclaving.

2YT medium: 10 g Bacto-yeast extract, 20 g Bacto-tryptone, 10 g NaCl and 2.5 g MgSO4 were dissolved in 1L of H_2O and the pH adjusted to 7.4 with 1M NaOH. This solution was sterilized by autoclaving.

Ψa medium: 5 g Bacto-yeast extract, 20 g Bacto-tryptone and 5 g MgSO4 were dissolved in 1L of H_2O. This solution was sterilized by autoclaving.

Agar plates (LB or Ψa): 15 g/L of Bacto-agar was added to the relevant medium solution prior to autoclaving. After this, the dissoved solution was slightly cooled, the relevant antibiotic solution was added if required, and poured into petri dishes in 12 ml aliquots. The plates were dried and stored at 4^0C.

Tfb I buffer: 0.58g KOAc, 1.491g KCl, 0.294g $CaCl_2.2H_2O$, 1.979g $MnCl_2.4H_2O$ and 30ml Glycerol was made up to 200ml with H_2O and adjusted to pH 5.8 with 0.2M acetic acid. This solution was sterilised through a 0.22μM filter.

Tfb II buffer: 0.21g MOPS (Sigma, USA, Cat. No. M-9381), 1.1g $CaCl_2.2H_2O$, 0.075g KCl, 15ml Glycerol was made up to 200ml with H_2O and adjusted to pH 6.5 with 0.5M KOH. This solution was sterilised through a 0.22μM filter.

A1.4 Reagents for DNA sequencing.

46% Urea: 460 g Urea (BDH, AnalR, Cat. No. 10290) was dissolved in 1L MQ H_2O. 20 - 30 g of Mixed Resin AG501-X8 (Biorad, Cat. No. 142-6424) was added and

stirred for 30 minutes. The solution was stored with the resin sediment and decanted for use.

10x TBE: 121 g Tris, 7.4 g EDTA and 53.4 g boric acid was added to 1L of MQ H2O and the pH was adjusted to 8.3.

20% Acrylamide solution: 467 g urea was added to 500 ml of a stock solution of 40% acrylamide (19:1, acrylamide : N, N'-methylene-bisacrylamide (Bio-Rad, Cat. No. 161-0144)) in H_2O. The volume was made up to 1L with MQ H_2O and the urea dissolved by stirring. 20 - 30 g of Mixed Resin AG501-X8 was added and stirred for 30 minutes. The solution was filtered through 3M Whatman paper and stored at RT in the dark.

A1.5 Reagents for Southern Blotting.

10x SSC: 87.65 g NaCl and 44.1 g sodium citrate was dissolved in H_2O to a final volume of 1L after adjusting to a pH 7 with NaOH. (where 1 x SSC is 0.15M NaCl and 0.015M sodium citrate)

50x Denhardt's: 5 g of Ficoll, 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin was added to 500 ml H_2O and filtered through a

Herring sperm DNA: Genomic DNA extracted from herring sperm was sheared by passing through a 25g needle and syringe several times after which it was boiled for 10 minutes.

Prehybridization solution: 50ml formamide, 25ml of 20x SSC (see above), 1ml of 0.2M sodium pyrophosphate pH7, 10ml of 50x Denhardts (see above), 1ml of 10% SDS, 10ml of 0.1M Hepes and 1ml of 0.1M EDTA were mixed and made up to a final volume of 100ml with MQ-water. Herring sperm DNA (see above) was added to a final concentration of 50ug/ml

A1.6 Reagents for Western Blotting.

100x Protease Inhibitors: Each of the following protease inhibitors were added to a final concentration of 4 mg/ml in dimethylformamide: TPCK (L-[1-tosylamido-2-phenyl] ethyl chloromethylketone), TLCK (1-chloro-3-tosylamido-7-amino-L-2-heptanone) and NPGB (p-Nitrophenyl-p'-guanidino-benzoate-HCl) and the solution was stored at -20^{0}C. The protease inhibitors were thawed immediately prior to use.

50x PMSF: 4mg/ml of PMSF was dissolved in ethanol to a final concentration of and stored similarly to the other protease inhibitors as above.

Lysis buffer: 2 ml 10% SDS, 1 ml glycerol and 1.2 ml 0.5M Tris-HCl pH 6.8 was mixed together and made up to 10 ml with MQ H_2O.

Running Gel polyacrylamide mix: 2.7ml of 30% Acrylamide, 2.5ml of 1.5M Tris-HCl pH 8.8, 100µl of 10% SDS, 100µl of APS, 6µl of TEMED and 4.6ml MQ water.

Stacking Gel polyacrylamide mix: 1.15ml MQ-water, 330µl 30% Acrylamide, 500µl 0.5M Tris-HCl pH 6.8, 20µl 10% SDS, 20µl APS and 2µl TEMED

Reducing 2x Loading buffer: 2 ml 0.5M Tris-HCl pH 6.8, 1.6 ml glyceral, 3.2 ml 10% SDS in H_2O, 0.8 ml β-mercaptoethanol and 0.4 ml 0.05% bromophenol blue were added together, aliqoted and stored at -20^{0}C

Running buffer (SDS-Page buffer): 3.03g Tris 1g SDS and 14.41g glycine were dissolved in 1L of H_2O and the pH adjusted to 8.3.

Transfer buffer (for submerged electrophoretic transfer apparatus) : A solution of 0.025mM Tris-HCl pH 8.3, 0.192mM glycine and 20% methanol in H_2O adjusted to pH 8.3.

10x TBS: 24.2g Tris and 87.6g NaCl were dissolved in 1L of H_2O and the pH adjusted to 7.5.

ADDITIONAL NOTES

General Points:

- Definition of Transformation: "Transformation" (Chapter 3) was assessed by the ability to form colonies in methylcellulose. "Complete Transformation" (Chapter 5-7) was used to define cells that were clonogenic as well as factor-independent and tumourigenic.
- Chapter 5 (section 5.4, pg 122-3): There is only indirect evidence that the cells were co-infected with V816Kit and CT3Myb. The colony assay (Figure 5.10) showed that co-infected cells were able to form factor-independent colonies compared to single infected cells that were not clonogenic in the absence of factor (Fgure 5.4A and Figure 5.10).
- Chapter 6: All the results (Figures and Table) show data from single experiments. All experiments were repeated and the result shown in each figure is representative of the overall data. Unusual experiments were discussed in Section 6.7.
- In all experiments with transfected or infected cells, pooled populations were used except in cases where individual colonies were shown.

Specific Points:

- pg 32: Seminomas are metastatic spermatogonial (germ cell) tumours.
- pg 86 (Section 3.2.4): Transformation was measured as the clonogenic potential of the infected cell populations following 7 days culture in GM-CSF.
- pg 113: relating to the statement "The cells in clusters were of varying size and appeared more differentiated ..." Cell smears of the colonies were analysed by staining with Giemsa.
- Figure 6.6e and 6.7e: Some apoptotic cells were observed in the population. It is possible that Kit could induce an apoptotic effect in cells, however further experiments would be necessary to investigate this possibility.
- pg 165 Ishii et al (1992): The title of the article is "Transcriptional control by the myb oncogene product" published in Tokohu J. Exp. Med., 168: 189-194.

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