THE TRANSFORMING POTENTIAL AND FUNCTIONAL ANALYSIS OF THE c-KIT RECEPTOR TYROSINE KINASE AND ITS NATURAL OCCURRING ISOFORMS.

By Georgina Caruana, B.Sc. (Hons.), Department of Microbiology and Immunology,
The University of Adelaide.

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ABSTRACT

In this study, the function of the receptor tyrosine kinase, c-Kit, was examined in relation to the role of receptor levels in factor dependence and cell transformation. In addition, the functions of several naturally occurring isoforms of the human c-Kit receptor were analysed by expressing cDNA encoding these isoforms in murine cells.

Cells of the murine factor-dependent cell line, FDC-P1, require the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) for cell growth. FDC-P1 cells also express c-Kit, however when cultured in the presence of its ligand, *Steel* factor (SLF), they fail to proliferate. Co-culture of FDC-P1 cells on wild-type (+/+) fibroblastoid-3T3 lines, in the absence of exogenously added factors, enabled contact-dependent support of the FDC-P1 cells in the presence of a neutralising antiserum to GM-CSF. +/+ fibroblasts are able to produce both membrane-bound and soluble forms of SLF. The lack of support of FDC-P1 cells by *Sl/Sl*^{d}-3T3 and *Sl/Sl*-3T3 fibroblasts, which produce only soluble SLF or completely lack SLF production, respectively, in the presence of GM-CSF antiserum, demonstrated that membrane-bound SLF was supporting FDC-P1 cells on +/+ fibroblast feeder layers.

Although FDC-P1 cells, which had been previously cultured in GM-CSF or IL-3, failed to proliferate in high concentrations of soluble murine SLF, the cells displayed synergistic proliferative responses to SLF when combined with sub-optimal levels of GM-CSF or IL-3. FDC-P1 cells previously grown in GM-CSF were adapted to grow in SLF alone by gradual substitution of SLF for GM-CSF over a period of 3 weeks. The resulting population, FDC-P1(SLF) cells, were responsive to SLF alone and maintained the ability to grow in GM-CSF or IL-3. mRNA analysis demonstrated that c-*kit* had been downregulated approximately 7-fold or 2.5-fold in the cells grown in GM-CSF or IL-3, respectively, as opposed to the cells grown in SLF alone (FDC-P1(SLF)). Downregulation of surface protein was also seen in FDC-P1 cells grown in GM-CSF or IL-3 compared with cells grown in SLF. Conversely, proliferation assays suggested that the GM-CSF and IL-3 receptors were not functionally downregulated by SLF, since FDC-P1(SLF) cells were able to proliferate just as well in GM-CSF or IL-3 as the cells originally grown in the latter factors. The differential

proliferative response of FDC-P1 cells to soluble SLF may be a consequence of the level of c-*kit* expressed by these cells.

Elevated receptor levels have been implicated in carcinogenesis. Overexpression of the receptor tyrosine kinase encoded by the *Her2/neu* proto-oncogene has been shown to be a prognostic indicator in a subset of breast and ovarian cancers. Similarly, Ashman *et al.*, (1988) demonstrated that high levels of the c-Kit receptor in a sub-group of acute myeloid leukaemic (AML) patients at presentation also correlated with poor prognosis. The remainder of this study therefore focussed on the potential role of the wild type c-Kit receptor in transformation, with respect to c-Kit receptor levels and their effect on factor dependence.

Ectopic expression of murine c-Kit in NIH/3T3 cells was achieved by infection with the defective retroviral vector, pZenneo, containing c-*kit* *c*DNA. Overexpression of the receptor demonstrated that the pool population of infectants, NIH(mu*kit*), grew in an anchorage independent, though factor-dependent manner in semi-solid agar, with a plating efficiency of 11% in 200 ng/ml of murine SLF. In the absence of exogenous SLF colonies appeared at a plating efficiency of 1%, possibly due to autocrine production of SLF by NIH/3T3 cells. Attempts to block endogenously produced SLF from binding to surface c-Kit, using the antagonistic anti-c-Kit monoclonal antibody, ACK2, or by neutralising SLF production with an anti-SLF antiserum revealed that the colonies were resistant to inhibition, implying that an intracellular interaction was occurring between c-Kit and SLF. The cells giving rise to these colonies expressed functional c-Kit receptors, demonstrated by their ability to reproduce SLF-dependent anchorage independent colonies upon replating in semi-solid agar. Analysis of the transformation potential of 24 clones spanning a 16.8 fold range of c-*kit* mRNA expression revealed a correlation between c-*kit* expression and anchorage-independence in the presence of 100 ng/ml SLF ($R = 0.53$; $p<0.01$). Cells expressing low levels of c-*kit* mRNA were mostly unable to produce anchorage independent colonies, in contrast to clones expressing medium to high levels of c-*kit* mRNA relative to the pool of NIH(mu*kit*) infectants. In the absence of exogenously added SLF only two clones, which also expressed high levels of c-*kit*, demonstrated significant anchorage independent

colony production. An increase in murine c-*kit* expression in early passage (ep) NIH/3T3 cells also resulted in enhanced transformation and tumourigenicity.

Similar studies were performed with the human c-Kit receptor and several of its natural occurring isoforms. One pair of isoforms differ in the deletion/insertion of 12 base pairs ($12^{-}/12^{+}$), encoding the amino acids Gly-Asn-Asn-Lys (GNNK), located in the extracellular domain adjacent to the transmembrane domain as a result of mRNA alternative splicing. A second set of mRNA splice variants exist, resulting in c-Kit isoforms which differ in the deletion/insertion of a serine residue (S^{-}/S^{+}), at position 715 located within the interkinase domain. Four c-*kit* cDNAs encoding the following isoforms were generated: *Kit(GNNK^{+}S^{+})*, *Kit(GNNK^{-}S^{+})*, *Kit(GNNK^{+}S^{-})*, *Kit(GNNK^{-}S^{-})* and cloned into the defective retroviral vector, pRUFMC1*neo*. Infection of epNIH/3T3 cells with these constructs and subsequent analysis of the ability of the c-Kit isoforms, expressed at comparable levels, to transform these cells, revealed discernible differences in their transforming potentials. c-Kit(GNNK-S+) was the most potent able to produce focus-formation, anchorage independent growth and induce tumours in *nude* mice. c-Kit(GNNK+S+) displayed the ability to produce focus-formation and anchorage independent growth, while c-Kit(GNNK+S-) was only able to induce focus-formation. Unfortunately, c-Kit(GNNK-S-) protein expression was not detected and as a result its transforming potential was not analysed. Analysis of cells expressing increasing levels of the isoforms demonstrated that focus-formation increased with an increase in c-Kit expression in a factor-dependent manner. epNIH/3T3 cells expressing c-Kit(GNNK-S+) at all levels and cells expressing c-Kit(GNNK+S-/+) at high levels were also able to induce focus-formation in the absence of exogenously added human SLF. In contrast to the focus-formation results cells expressing increasing levels of the human c-Kit isoforms demonstrated that, surprisingly, as the level of c-Kit increased, the number of anchorage independent colonies decreased in the presence and absence of human SLF. The latter was also demonstrated in an independent system in which the c-*kit(GNNK^{+}S^{+})* cDNA was cloned in a mammalian expression vector pRSV009/A^{+}. This vector encodes a dihydrofolate reductase gene, allowing sequential amplification of the linked c-*kit* gene upon increasing concentrations of methotrexate selection in NIH/3T3 cells. It appeared that a

'window' of c-Kit receptor level expression was required for maximal induction of transformation.

Constructs encoding the c-Kit isoforms were also introduced into murine factor-dependent cell lines. Cells expressing comparable levels of the c-Kit receptor were analysed for their responsiveness to SLF, and the ability of a range of monoclonal antibodies to c-Kit, to block SLF-driven proliferation.

STATEMENT

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

Georgina Caruana

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ABBREVIATIONS

aa	amino acid
AML	Acute Myeloid Leukaemia
amp	ampicillin
APAAP	Alkaline phosphatase anti-alkaline phosphatase
ATCC	American Tissue Culture Collection
Az	Azide
BFU-E	Erythroid-burst forming unit
BFU-Mk	Megakaryocyte-burst forming unit
bio	biotin-labelled
BMMC	Bone marrow derived mast cells
bp	base pairs
BSA	Bovine serum albumin
c-*onc*	cellular oncogene
cDNA	complementary DNA
CFU-GEMM	Multipotential- colony forming unit
CFU-GM	Granulocyte-macrophage colony forming unit
CFU-S	Spleen -colony forming unit
CIP	Calf intestinal phosphatase
COOH	carboxyl- terminus
cpm	counts per minute
CSF-1 (R)	Colony-stimulating factor- 1 (Receptor)
DEPC	Diethyl pyrocarbonate
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's Modified Eagle's Medium
DTT	1,4-Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
EGF (R)	Epidermal growth factor (Receptor)

ep	early passage
Epo	Erythropoietin
FBS	Fetal bovine serum
FGF (R)	Fibroblast growth factor (Receptor)
FITC	Fluorescein-isothiocyanate
GαM	Goat anti- mouse
G-CSF	Granulocyte colony-stimulating factor
G418	Geneticin
GAPDH	Glyceraldehyde 3- phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GNNK	Glycine-Asparagine-Asparagine-Lysine
h	human
HBS	Hepes buffered saline
HBSS	Hank's balanced salt solution
HEPES	N-2-Hydroxyethylpiperazine N'2-ethane sulphonic acid
HZ4-FeSV	Hardy Zuckerman 4-Feline Sarcoma Virus
IFNγ	Interferon- gamma
Ig	Immunoglobulin
IGF-1 (R)	Insulin-like growth factor receptor
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IIF	Indirect immunofluorescence
IL-3,-4,-7,-6, -9,-10,-11,-12	Interleukin-3, -4,-7,-6, -9,-10,-11,-12
IMDM	Iscove's Modified Dulbecco's Medium
IMVS	Institute of Medical and Veterinary Science
IRR	Insulin Receptor
KI	Kinase insert
LB	Luria Broth
LTBMC	Long term bone marrow culture

LTR	Long terminal repeat
m	murine
mAb	Monoclonal Antibody
MAPK	Mitogen-activated protein kinase
mfi	Mean fluorescence intensity
MNC	Mononuclear cells
mRNA	messenger RNA
Mtx	Methotrexate
mwt	Molecular weight
NDF	Neu differentiation factor
neo	neomycin
NH2	amino-terminus
NK	Natural killer
NRS	Normal rabbit serum
NSS	Normal sheep serum
OPC	Oligonucleotide Purification Column
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF (R)	Platelet-derived growth factor (Receptor)
PE	Phycoerythrin
PI	Propidium iodide
PI 3'-K	Phosphatidylinositol 3'-kinase
PKC	Protein kinsase C
PLC-γ1	Phospholipase C- gamma 1
PNK	Polynucleotide kinase
RαG	Rabbit anti-goat
RBC	Red blood cells
RSV	Rous Sarcoma Virus
RT	Room temperature

RTK	Receptor tyrosine kinase
S	Serine
SDS	Sodium dodecyl sulfate
Sl	*Steel*
SH2-3	Src- homology 2-3
SLF	*Steel* factor
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TC	Tissue culture
TE	Tris-EDTA
TGF-α	Transforming growth factor-alpha
v-*onc*	viral oncogene
VEGF (R)	Vascular endothelial growth factor (Receptor)
W	*White spotting*

CONTENTS

CHAPTER 2 : MATERIALS AND METHODS

CONFERENCE PRESENTATIONS

1993 **5th Lorne Cancer Conference, Victoria, AUSTRALIA**

Poster Title: Functional Regulation of the c-Kit Receptor in the Murine Myeloid Cell Line FDC-P1 by GM-CSF and IL-3.

G.Caruana, L.K. Ashman, and T.J. Gonda.

1994 **6th Lorne Cancer Conference, Victoria, AUSTRALIA**

Poster Title: Overexpression of the c-*kit* Proto-oncogene Causes Transformation in NIH-3T3 Cells

G.Caruana, T.J. Gonda, and L.K. Ashman.

1996 **Keystone Symposia Conference on Signal Transduction through Tyrosine Kinases**

Poster Title: Functional Studies of Isoforms of the Human c-Kit Receptor Tyrosine Kinase.

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PUBLICATIONS

Caruana, G., Ashman, L.K., Fujita, J., and. Gonda, T.J. (1993) Respones of the murine myeloid cell line FDC-P1 to soluble and membrane-bound forms of steel factor (SLF). Experimental Hematology 21: 761-768.

Aylett, G. W., Cole, S. R., **Caruana, G.**, and Ashman, L. K. (1995). Specificity and functional effects of monoclonal antibodies to the c-Kit protein (SCF receptor). In : Leucocyte Typing V : White Cell Differentiation Antigens. Proceedings of the Fifth International Workshop and Conference. Vol. II. edited by Schlossman *et al.*, Published by Oxford University Press.

Publications in Preparation

The Effect of c-Kit Receptor Density in the Induction of Transformation of NIH/3T3 cells.

Caruana, G., Cambareri, A. C., and Ashman, L. K.

The Transforming Potential of Isoforms of the Human c-Kit Receptor Tyrosine Kinase.

Caruana, G., and Ashman, L. K.

CHAPTER 1

INTRODUCTION

1.1 The Capture of proto-oncogenes by Retroviruses

The discovery that neoplastic growth induced by acute transforming retroviruses, was a result of the activation of host cellular genes, known as "proto-oncogenes," led to the discovery of a wide range of proteins which are involved in the normal processes of cellular proliferation and differentiation. Cellular oncogenes (c-*onc*) or proto-oncogenes are not tumourigenic in their normal state but require activation to abnormal levels of expression and/or mutated so as to change some aspect of their function. The ability of retroviruses to pick up these cellular sequences and transduce them into oncogenes (v-*onc*), able to produce tumours in their host, has enabled the cloning of these genes which may otherwise still be unknown to us today.

In 1911, Peyton Rous, demonstrated the transmission of sarcomas by cell-free extracts of chicken sarcomas and identified the infectious agent as a virus. This virus was later identified as a RNA tumour virus or retrovirus known as the Rous Sarcoma Virus (RSV) and has become the paradigm of all retroviruses (for review see Coffin, 1982). Many retroviruses have been isolated from a variety of animal species including: mice, chickens, turkeys, rats, cats, woolly and gibbon monkeys. The study of these retroviruses has enabled their classification into 3 groups according to their biological properties, morphology and genomic structure. These include the complete-transforming, slow-transforming and replication-defective transforming viruses.

The RSV belongs to the complete-transforming retroviral group. It is able to induce tumours rapidly and at a high frequency. The RSV contains all its own replication machinery. The retrovirus genome structure consists of two RNA strands each containing 3 viral genes (1) the protein capsid (*gag* = group-specific antigen) (2) RNA-dependent DNA polymerase, "reverse transcriptase" (*pol*) and (3) the envelope antigens (*env*), which are embedded in the membrane surrounding the viral capsid, determining host range and mediating virus binding to the host cell during infection. RSV also possess another gene which renders the virus able to induce tumours (Martin, 1970; Vogt 1971; Bernstein *et al.*, 1976; Stehelin *et al.*, 1976a). This oncogene is known as v-*src*, for sarcoma and was found to be derived from a host cellular gene c-*src* (Stehelin *et al.*, 1976b).

Once the virus infects the host cell the single stranded RNA genome is transcribed into a double-stranded linear DNA molecule using the viral reverse transcriptase. At the 5' and 3' ends of the DNA are specialised sequences of 300-1000 base pairs known as long terminal repeats (LTR). The LTR are derived from a combination of sequences present at the 3' end (U_3), 5' end (U_5) and a redundant region (R) at both extremities of the RNA genome, and has the structure U_3-R-U_5. Once synthesised, the proviral DNA integrates into the host genome. Each successfully infected cell contains 1-several integrated proviruses. The LTR sequences are required for integration and a 5-base pair sequence of host DNA is directly repeated at each end of the provirus. Integration appears to be random and the orientation of the provirus is exactly the same as for the free linear viral DNA. The LTR are directly joined to cellular sequences and, once the provirus has integrated, they act as promoter and enhancer elements. Subsequent steps in viral replication are carried out by the host cell. Viral RNA is synthesised using cellular RNA polymerase II. The transcripts serve as mRNA for virial proteins (products of *gag*, *pol* and *env*) or for new RNA genomes. Signals directing efficient transcription of the provirus are present in the U_3 portion of the LTR. Once transcription has occurred, the viral proteins are combined with single stranded diploid RNA genomes to yield complete virions. Fully assembled infectious progeny virus buds from the cell surface, the envelope of the virion being formed at the cellular plasma membrane as virus is released from the host cell, without killing it (Varmus *et al.*, 1982).

Slow-transforming retroviruses induce cancer after a long latency period. Understanding of the mechanism of induction of transformation by these retroviruses came from studying B cell leukaemias induced by avian leukosis (ALV) in chickens. The tumours induced by this virus contain viral DNA integrated in common domains of the host genome. Within the vicinity of the integration site lies a cellular gene whose activity is augmented as a consequence of the insertion of the viral DNA. In most cases the oncogenesis in B cells induced by ALV is due to the insertional activation of the cellular oncogene c-*myc*. The virus has usually under gone a deletion often near to or including the 5' LTR, therefore the 3' LTR is often used as the promoter. Thus c-*myc* is activated due to promoter insertion and the level of c-*myc* RNA can be 10 to 50 times higher than normal levels (Bishop, 1983).

Replication-defective transforming retroviruses as their name suggests are defective in one or more of the viral genes required for replication. In order for these viruses to replicate they require helper virus that is able to supply the missing viral gene products. Nevertheless these retroviruses are able to induce tumours rapidly and at a high frequency. As with the RSV, these retroviruses have picked up normal cellular gene sequences which enable them to produce tumours, however the capturing of these sequences disrupts the genes required for viral replication. The most commonly disrupted is the *env* gene, but deletions of all or part of the *gag* and *pol* genes have also been seen, generally resulting in part of the normal cellular gene being fused to the *gag* gene (Watson *et al.*, 1987a).

The mechanisms of cellular transduction by retroviruses can be explained by the following model: the first step requires the integration of the provirus upstream of a proto-oncogene. A recombination event may occur that results in the deletion of the 3' LTR and part of the proviral sequences replacing them with part of the proto-oncogene sequence. This DNA would be transcribed from the viral 5' LTR to mRNA with the introns of the cellular sequence being spliced out. This RNA molecule then dimerizes with other defective or normal RNA viral genome molecules ready for packaging. At the 5' end of the RNA molecule is a packaging sequence (Psi) which allows packaging of the native and hybrid RNA genomes into virions supplied by the co-infecting helper virus. The viral genes *gag*, *pol* and *env* are provided by the helper virus in the case of replication-defective transforming retroviruses. In order for the defective retrovirus to reinfect a cell and the provirus to integrate into the host genome, the transduced proto-oncogene must also recombine with the 3' end of a viral genome. 3' recombination may occur during reverse transcription ie. between RNA molecules after packaging and before the next round of proviral integration into newly infected cells. These 5' and 3' recombination events result in part of the proto-oncogene sequence to be deleted causing activation, rendering it oncogenic. Most genes transduced by retroviruses have errors at both the 5' and 3' ends. However other events during the retroviral life cycle can disrupt the proto-oncogene and activate it. Errors can be introduced during reverse transcription, at each replication cycle, as many as 0.5% of viral RNA sequences reverse transcribed to DNA are copied incorrectly. Though many of these errors produce

non-viable viruses or inconsequential changes, there are the rare base changes that result in producing a transforming oncogene (v-*onc*) (reviewed in Bishop, 1983; Watson *et al.*, 1987a and references therein).

The discovery that the v-*onc* harboured by retroviruses where derived from proto-oncogenes (c-*onc*) was first revealed through the analysis of v-*src*. DNA encoding the v-*src* gene from the RSV genome was found to hybridise to DNA from uninfected chickens and several other avian species as well as to human, calf, mouse and salmon DNA (Stehelin *et al.*, 1976b; Spector *et al.*, 1978). Subsequent cloning of the cellular gene and sequence analysis revealed that the viral oncogene v-*src*, contained within the retroviral genome, differed from the cellular gene, c-*src,* from which it was derived. Both v-*src* and c-*src* proteins had molecular weights of 60 kd (Brugge *et al.*, 1977; Oppermann *et al.*, 1979) however, v-*src* contained 8 point mutations changing amino acids within its protein sequence compared to c-*src*. Other changes involved the substitution of the last 19 amino acids at the carboxyl terminus of the cellular protein with a different sequence of 12 amino acids acquired from downstream of the c-*src* locus by DNA rearrangement (Swanstrom *et al.*, 1983; Takeya *et al.*, 1983). The expression of v-*src* was much higher than the levels of endogenous c-*src* within uninfected cells and it was initially suggested that this overexpression of v-*src*, due to transcriptional activation under the strong LTR promoter, may be the reason for neoplastic growth. However, experiments showed that chicken c-*src* when expressed at up to 10-15 fold higher levels than the transforming levels of v-*src* was unable to induce a fully transformed phenotype in chicken or rodent cells *in vitro* (for description of *in vitro* transformation assays see section 1.9.1). Also, if v-*src* expression was reduced to levels comparable with those of endogenous c-*src,* it was still able to induce transformation, suggesting that overexpression of this gene was not enough to disrupt its normal function within the cell (Iba *et al.*, 1984; Parker *et al.*, 1984; Shalloway *et al.*, 1984). It was therefore most likely that the mutational differences between v-*src* and c-*src* were the cause for inducing transformation. This was in contrast to the work involving unaltered c-*mos*, c-*fos,* c-*ras* (Blair *et al.*, 1981; Chang *et al.*, 1982; Miller *et al.*, 1984). When these cellular oncogenes were placed under the control of a

retrovirus transcriptional promoter to produce high levels of protein expression, the transfected cells assumed a fully transformed phenotype.

As well as v-*src* being expressed at much higher levels in transformed cells compared to endogenous c-*src* in uninfected cells, it was also more highly phosphorylated. The v-*src* gene product was found to be a phosphoprotein designated, pp60$^{v\text{-}src}$, (Collett *et al.*, 1978; Levinson *et al.*, 1978) attached to the inner surface of the plasma membrane by myristic acid modification of the NH_2 terminus (Cantley *et al.*, 1991). Both v-*src* and c-*src* gene products were found to possess a novel protein-tyrosine kinase activity (Hunter *et al.*, 1980) which enabled them to catalyse the transfer of the terminal phosphoryl group of ATP to the hydroxyl group of a tyrosine residue in its substrates. pp60^{src} also possesses serine/threonine kinase activity and two domains referred to as Src-Homology 2 and 3 (SH2 and SH3) which have been shown to act as sites able to interact with other cytoplasmic proteins involved in signal transduction pathways (Koch *et al.*, 1991). The higher levels of phosphorylation associated with v-*src* suggested that the mutations were able to enhance its kinase activity and that this may contribute to its neoplastic transformation ability (Collett *et al.*, 1978; Sefton *et al.*, 1980). The tyrosine residue 416 (Y_{416}) is phosphorylated in pp60$^{v\text{-}src}$ (Smart *et al.*, 1981). Y_{527} situated in the carboxyl tail of pp60$^{c\text{-}src}$, suppresses kinase activity upon phosphorylation (Courtneidge 1985; Cooper *et al.*, 1986). Dephosphorylation and mutagenesis of Y_{527} results in phosphorylation of Y_{416} increasing the kinase activity of pp60$^{c\text{-}src}$ enabling it to induce transformation (Cantley *et al.*, 1991 and references therein). It has been shown that the reduction in protein-tyrosine kinase activity of pp60$^{c\text{-}src}$ correlates with an intramolecular association between phosphorylated Y_{527} and the SH2 domain of the protein, resulting in a "head-to-tail" self-association of the molecule (Roussel *et al.*, 1991). Y_{527} is missing in pp60$^{v\text{-}src}$ due to the carboxyl deletion, therefore the lack of phosphorylation at this residue and/or mutations within the SH2 domain contribute to the transforming ability of pp60$^{v\text{-}src}$. These results imply that Y_{527} serves as a negative regulatory site of phosphorylation in pp60$^{c\text{-}src}$ whereas phosphorylation of Y_{416} correlates with the activation of kinase activity (Koch *et al.*, 1991; Roussel *et al.*, 1991; Cantley *et al.*, 1991 and references therein).

The discovery that v-*src* catalyzes the phosphorylation of proteins on tyrosine was of great important since many other oncogenes transduced by retroviruses were also found to possess areas of sequence homology corresponding to the *src* tyrosine kinase catalytic domain. The cellular counterparts of these oncogenes have been shown to encode either transmembrane proteins, such as the receptor tyrosine kinases (RTK) (c-*erbB1*, c-*fms*, c-*kit*) (see section 1.2) or cytoplasmic proteins like c-*src* (c-*yes*, c-*abl*, c-*fgr*, c-*fps*) which are also involved in signal transduction pathways (Cantley *et al.*, 1991). As has been discussed briefly with *src* and will be discussed in more detail with several members of the RTK family throughout this chapter, the mutations inflicted by retroviruses on these genes affect the normal function of these molecules. Many of the mutations are directly or indirectly targeted toward disrupting the regulated protein tyrosine kinase activity of these receptors, which in turn disrupts the cascade of events that normally occurs upon ligand binding to these receptors, and results in tumourigenesis.

Many proto-oncogenes captured by retroviruses have been shown to be highly conserved through evolution indicating that they play essential roles in normal cell processes. The proto-oncogenes discovered thus far can be catergorised into groups according to their similarities in amino acid sequences, similar enzymatic activity eg tyrosine or serine/threonine kinase activity or intracellular location; a few of these are summarised in Table 1.1. The oncogenic activation of many of these genes has not only aided in discovering the role they play in cancer but has also aided in discovering how these genes normally function in the cell; as Michael Bishop once said "in studying the abnormal, we also learn of the normal" (1991). The remainder of this chapter will focus mainly on the proto-oncogene unveiled to us by the Harvey-Zuckerman 4 Feline Sarcoma Virus (HZ4-FeSV) carrying part of the cellular sequences of the c-*kit* gene.

1.1.1 The discovery of the c-*kit* proto-oncogene

Infectious filtrate obtained from a primary feline fibrosarcoma was shown by Besmer *et al.* (1986b) to transform feline embryo fibroblasts and mink cells. A feline leukaemia virus (FeLV) belonging to the Hardy-Zuckerman 4 strain (HZ4-FeSV) was identified as the

TABLE 1.1 : The Captured Proto-oncogenes of Retroviruses[∞]

oncogene	retrovirus	v-*onc* origin	proto-oncogene function
Protein kinases			
src	Rous Sarcoma Virus (RSV)	chicken	tyrosine-specific kinase
yes	Y73- avian sarcoma virus (ASV)	chicken	tyrosine-specific kinase
fgr	Gardner-Rasheed feline sarcoma virus (FeSV)	cat	tyrosine-specific kinase
abl	Abelson murine leukaemia virus Hardy-Zuckerman (HZ2)-FeSV	mouse cat	tyrosine-specific kinase
fps/fes	Fujinami sarcoma virus (FuSV)	chicken	tyrosine-specific kinase
	PRCII-ASV	chicken	
	Synder-Theilen-FeSV	cat	
mos	Moloney murine sarcoma virus(MSV)	mouse	component of CSF/serine/threonine protein kinase
raf/mil	3611-MSV	mouse	serine/threonine protein kinase
ros	UR II -ASV	chicken	receptor/tyrosine-specific protein kinase
erb B	Avian erythroblastosis (AEV)	chicken	EGFR/tyrosine-specific protein kinase
fms	McDonough (SM)-FeSV	cat	CSF-1R/tyrosine-specific protein kinase
	HZ5-FeSV		
kit	HZ4-FeSV	cat	c-KitR/tyrosine-specific protein kinase
GTP binding proteins			
H-ras	Harvey MSV (Ha-MSV)	rat	guanine nucleotide binding protein with GTPase activity
K-ras	Kirsten MSV (Ki-MSV)	rat	guanine nucleotide binding protein with GTPase activity

Growth factors			
sis	Simian sarcoma virus (SSV) Parodi-Irgens FeSV	woolly monkey cat	analogue of PDGF-B
Nuclear proteins			
myc	Avian MC29 myelocytomatosis virus	mouse	transcription factors
myb	Avian myeloblastosis virus (AMV)	chicken	transcription factors
	AMV-E26	chicken	
fos	FBJ-MSV	mouse	transcription factors
ski	Avian SKV770 virus	chicken	transcription factors
rel	Reticuloendotheliosis virus (REV)	turkey	NF-kappa B-like transcription factor
ets-1	AMV-E26	chicken	transcription factor
Hormone receptor			
erbA	AEV	chicken	thyroid hormone receptor
Unclassified			
**cbl*	murine Cas NS-1 leukaemia virus	mouse	involved in tyrosine-kinase mediated signal transduction

∞Table 1.1 includes a selection of proto-oncogenes transduced by complete or replication-defective transforming retroviruses and is a modification of tables included in Bishop 1985, 1991 and Watson *et al.*, 1987a.

* Andoniou *et al.*, (1994) and Galisteo *et al.*, (1995)

transforming infectious agent. The transformed mink cells were unable to produce infectious HZ4-FeSV, however the retrovirus could be rescued by superinfection with the amphotrophic murine leukaemia virus (MuLV) (ie. a helper virus). This catergorised the HZ4-FeSV into the group of retroviruses known to be replication-defective (see section above), suggesting that this transforming retrovirus may be harbouring an oncogene which was disrupting viral-replication. Restriction fragment analysis of the HZ4-FeSV provirus, obtained from mink infected cells, showed this to be the case revealing a *Sac* / *Sal* fragment which lacked homology with FeLV sequences. This non-viral sequence specific to the HZ4-FeSV was designated v-*kit*. Protein analysis using antisera specific for the gag protein, p27, of FeSV, immunoprecipitated an 80 kd protein, p80, suggesting that the *gag* and v-*kit* products form a fusion protein. The structure of the HZ4-FeSV provirus was 5' Δgag-kit-Δpol-Δenv 3', the v-*kit* insert being 1.1 kb in size encoding 370 amino acids. Since v-*kit* lacks both an extracellular and transmembrane domain, it is attached to the membrane via gag-linked myristoylation (common factor to *src* gene product). In order to determine whether it was derived from a cellular gene, v-*kit* sequence was used to probe cat, human and mouse DNA, and homologous sequences were found to be present in all cases, the transcript size ranging from 5 - 5.5 kb (Yarden *et al.*, 1987a; Qui *et al.*, 1988).

Cloning of the cellular counter-part of v-*kit* from both mouse and human cDNA libraries, obtained from brain and placenta respectively, revealed that structural differences existed between the v-*kit* and c-*kit* gene products. V-*kit* had been truncated at both the NH_2 and COOH termini during transduction resulting in a product which lacked the entire extracellular domain, the transmembrane domain, the first 17 amino acid residues of the intracellular domain, as well as the COOH-terminal 49-50 amino acid residues which were replaced with 5 unrelated amino acids. Other differences also existed between murine c-*kit* and v-*kit* involving 20 amino acids (Yarden *et al.*, 1987a; Qui *et al.*, 1988). All but three of these differences have been recently attributed to divergence between the species (Herbst *et al.*, 1995a). These three alterations have been shown to contribute to the oncogenicity of v-*kit* as is discussed further in section 1.9.3.1.

Human c-*kit* cDNA encodes a 976 amino acid polypeptide with a molecular weight of 145 kd (fully glycosylated; immature form = 125 kd) (Yarden *et al.*, 1987a) and therefore designated, p145$^{c\text{-}kit}$. p145$^{c\text{-}kit}$ (will be referred to as c-Kit from here on) shares major structural features to the macrophage colony-stimulating factor-1 receptor (CSF-1R), α and β platelet derived growth factor receptors (PDGFR) (Yarden *et al.*, 1987a; Qui *et al.*, 1988) and the recently cloned *flt*3/*flk*2 receptor (Matthews *et al.*, 1991; Rosnet *et al.*, 1991), which are all growth factor receptors belonging to the receptor tyrosine kinase type III (RTK III) family (see section 1.2) sharing sequence homology within their catalytic kinase domain with c-*src*. Despite their structural similiarities, the non-catalytic sequences of the three proteins (ie CSF-1R, PDGFR and c-Kit receptor) display sequence heterogeneity that is highest in the ligand binding domain and the kinase insert. These regions define the functional specificity of each receptor (discussed in section 1.7).

1. 2 Growth Factor Receptor Tyrosine Kinases

All growth factor receptors belonging to the receptor tyrosine kinase (RTK) family possess a large glycosylated extracellular ligand binding domain, a hydrophobic transmembrane domain, and a cytoplasmic domain that contains a tyrosine kinase catalytic domain. The human c-*kit* cDNA sequence predicts that the protein possesses a 23 amino acid (aa) signal peptide, 497 aa extracellular domain, 23 aa transmembrane domain and 433 aa intracellular domain (Yarden *et al.*, 1987a). Based on sequence and structural similiarilties, there are several subclasses of receptors possessing tyrosine kinase activity (reviewed in Hanks *et al.*, 1988; Yarden *et al.*, 1988; Ullrich & Schlessinger, 1990; Heldin 1995).

RTK type I includes the epidermal growth factor receptor (EGFR encoded by c-*erbB1*) and its close relatives HER-2/*neu*, HER-3 and HER-4 (encoded by c-*erbB2*, *3* and *4*, respectively). RTK type I receptors possess two cysteine-rich repeat sequences within the extracellular domain. RTK type II receptors include the insulin (IRR) and insulin-like growth factor (IGF-1) receptors which function as heterotetrameric structures involving two α and β subunits. The α subunits bind the ligand and are disulphide-linked to the β subunits which traverse the membrane and possess the tyrosine kinase domains. One cysteine-rich repeat

sequence is found on each α subunit. RTK type III include the CSF-1R (encoded by c-*fms*), α and βPDGFR, the *flt3*/*flk2* and c-Kit receptors. Members of this subclass do not have a cysteine-rich repeat sequence but instead possess 10 distinctively positioned cysteine residues which contribute to the formation of five immunoglobulin (Ig)-like domains within the extracellular region. RTK type III also differ in their intracellular region compared to the other subclasses in that their protein tyrosine kinase catalytic domain is interrupted by a poorly conserved, non-catalytic, hydrophilic insertion sequence of varying length (70-100 residues) (77 aa for c-Kit) and has been referred to as the kinase insert (KI) or interkinase domain. The RTK type IV sub-class is structurally similar to RTK III also containing a KI domain, but only possesses two-three Ig-like loops in its extracellular domain. Fibroblast growth factor receptors (FGFR) are examples of this group. Recently another subclass has emerged which also resembles RTK III but has 7 Ig-like loops in the extracellular domain. Members of this group include, *flk*1, *flt*1 and *flt*4, and members of the vascular endothelial growth factor (VEGF) receptor family. Additional RTK families also exist and new members are constantly being discovered (reviewed by Lemmon and Schlessinger, 1994; Heldin, 1995).

The RTK function upon ligand binding to the extracellular domain, this in turn leads to cross-linking of receptors by ligand and/or a conformational change within the receptors resulting in dimerization. Dimerization enables the cytoplasmic portions of the receptor subunits to come into close proximity in a stabilised fashion. Dimerization initiates autophosphorylation of the receptors on tyrosine residues which creates high-affinity binding sites for substrates involved in intracellular signalling pathways. Most of these substrates bind to activated receptors through a protein sequence known as the Src-homology-2 (SH2) domain that recognises phosphotyrosine residues in the context of a short sequence of amino acids. The diversity of receptor phosphorylation sites, together with a wide range of SH2-containing substrate molecules, provides the basis for the unique repertoire of signalling molecules for each receptor. Deciphering how the RTKs function has been aided by analysing the retroviral counter-parts of many of these receptors as well as naturally occurring (eg *W* mutant mice for the c-Kit receptor, see section 1.4) and site-directed generated mutants. The

individual events which occur upon activation of the RTKs, focussing on c-Kit, will be discussed in more detail later (see section 1.7).

1.3 Alternative splice variants of c-*kit*

In both mouse and human genomes c-*kit* spans over 80 kb, the coding sequence being distributed over 21 exons (André *et al.*, 1992; Giebel *et al.*, 1992; Gokkel *et al.*, 1992; Vandenbark *et al.*, 1992). The use of alternative 5' splice donor sites at the 3' end of exon 9 (5' to the exon encoding the transmembrane domain) gives rise to two alternatively spliced c-*kit* transcripts in both human and murine cell lines and tissues. These two isoforms differ by an in-frame deletion (Kit+) or insertion (KitA+) of 12 base pairs (bp) which encode for 4 amino acids Gly-Asn-Asn-Lys (minus or plus codons 510-513) in the extracellular domain just prior to the transmembrane domain (Hayashi *et al.*, 1991; Reith *et al.*, 1991b; Gokkel *et al.*, 1992; Vandenbark *et al.*, 1992) (refer to Chapter 5, Figure 5.3.1A for diagrammatical representation). From here on the KitA+ and Kit+ isoforms will be referred to as c-Kit(GNNK+) and c-Kit(GNNK-), respectively.

Both isoforms have been found to be co-expressed in mast cells, placenta, lung, fetal liver, ovary, testis, melanocytes, glioblastoma cell line, normal bone marrow cells, leukaemic cell lines and acute myeloid leukaemic (AML) cells, the c-Kit(GNNK-) isoform being the more dominantly expressed (Reith *et al.*, 1991; Giebel *et al.*, 1992; Crosier *et al.*, 1993; Piao *et al.*, 1994; Zhu *et al.*, 1994). Concerning the AML cells analysed no correlation was found between the levels of isoform expression and the aggression of the malignancy or with FAB classification.

Crosier *et al.,* (1993) described the existence of two other human c-*kit* isoforms, also the result of alternative splicing, involving alternative usage of the 3' splice acceptor site of exon 15. This results in the in-frame deletion or insertion of 3 bp encoding a serine at residue 715 (S_{715}) (refer to Chapter 5, Figure 5.3.1A for diagrammatical representation). The murine c-*kit* sequence (Qui *et al.*, 1988) does not contain an equivalent serine to S_{715}. Analysis of the genomic structure of murine c-*kit* illustrates that only one 3' acceptor site exists at the intron/exon junction of exon 15, that which produces the inclusion of S_{715} only exists in the

human genomic c-*kit* sequence. S_{715} resides in the interkinase domain of the c-Kit receptor, close to the binding site of the p85 subunit of phosphatidylinositol 3'-kinase (PI 3'-K), tyrosine residue (Y_{719}) of the mouse c-Kit receptor (Serve *et al.*, 1994), the equivalent tyrosine in the human receptor lies at position 721 (Shearman *et al.*, 1993). Analysis of the expression of these two isoforms in normal bone marrow cells, leukaemic cell lines and AML cells revealed that they are co-expressed with dominance of the S+ isoform (Crosier *et al.*, 1993).

The role of the c-Kit(GNNK+/-) and c-Kit(S+/-) isoforms have not been determined to date. However, deletion of the 12 bp in c-Kit(GNNK-) renders the receptor constitutively phosphorylated at a low level in the absence of ligand. Both isoforms have the ability to associate with PI 3'-K and phospholipase Cγ-1 (PLCγ-1) when stimulated with the c-Kit ligand, *Steel* factor (SLF), however c-Kit(GNNK-) is also able to associate with these signalling proteins in the absence of ligand (Reith *et al.*, 1991). Low levels of constitutive activation of the c-Kit(GNNK-) isoform may provide sufficient signalling to promote cell survival of progenitor cells, whereas higher levels of stimulation by SLF may be necessary to induce proliferation and/or differentiation (Williams *et al.*, 1992). It is possible that these receptors may also heterodimerize (see section 1.8.1) to regulate the signal through the c-Kit receptor or that their expression is altered during the differentiation of stem cells (Piao *et al.*, 1994). Other receptors such as HER-2/*neu* (Siegel *et al.*, 1994) and CD44 (Dougherty *et al.*, 1991; Günthert *et al.*, 1991) have been reported to possess isoforms which differ in sequences lying in the extracellular domain proximal to the transmembrane domain. These are also produced by alternative splicing and they may alter ligand binding and/or signalling and/or the oncogenic potential of the receptor. In mice additional variant c-*kit* transcripts are also expressed in gametogenesis (Sorrentino *et al.*, 1991; Rossi *et al.*, 1992).

1.4 Association of c-*kit* with the W locus

Using somatic cell hybrid and *in situ* hybridisation techniques *c-kit* was mapped to the human long arm of chromosome 4 between 4q11-q21 and murine *c-kit* to mouse chromosome 5 (Yarden *et al.*, 1987a). The location of murine *c-kit* on chromosome 5 led to the hypothesis

that *c-kit* may be linked to the *W* locus also found on this chromosome. Mutant mice carrying defects at the *W* loci (W^{19H} and W^{44}) were analysed for alterations in *c-kit* and, in the case of W^{44}, a rearrangement confined to the *c-kit* gene was found linking *c-kit* to the *W* locus (Chabot *et al.*, 1988; Geissler *et al.*, 1988). The existence of spontaneous *W* mutant mice provided an insight into the function of the c-Kit receptor. Many *W* mutant mice have been described thus far and display defects in: gametogenesis affecting fertility; melanogenesis resulting in lack of hair pigmentation or "white-spotting"; defects in haemopoiesis resulting in macrocytic anaemia and mast cell deficiencies (reviewed in Sarvella *et al.*, 1956; Kitamura *et al.*, 1978; Russell 1979) (see also Table 1.4.1A). DNA and protein analysis of c-*kit* from mast cells obtained from various *W* mutant mice have revealed structural alterations within the *c-kit* gene which range from genomic rearrangments, partial deletions and single point mutations (Geissler *et al.*, 1988; Nocka *et al.*, 1990b; Reith *et al.*, 1990). Kinase activity associated with the c-*kit* gene product was also affected in mast cells obtained from *W* mutant mice (Nocka *et al.*, 1989; Reith *et al.*, 1990). Demonstration that c-*kit* was expressed by the lineages affected in the *W* mutant mice, such as foetal and adult erythropoietic tissues, mast cells and neural-crest-derived melanocytes (Nocka *et al.*, 1989), as well as the data described above provided sufficient evidence that the c-*kit* gene and the murine *white spotting locus* (*W*) were allelic.

Mutations at the *W* locus represented the first examples of germ-line mutations in a mammalian proto-oncogene (Chabot *et al.*, 1988). The *W* mutant mice vary in the severity of their defects which parallels with the severity of the mutation within the c-Kit receptor and whether the mice are homozygous or heterozygous for the c-*kit* alleles. For the original *W* mutation, mice carrying one W allele (*W*/+) have a relatively mild phenotype displaying a ventral white spot, pigmentless feet and tail tip, but their blood parameters and fertility are normal. Mice carrying both *W* alleles (*W/W*) die neonatally or *in utero* of severe macrocytic anaemia (Little 1915). Table 1.4.1A summarises the phenotypes of several homozygous and heterozygous *W* mutant mice.

Many of the targeted mutations involve conserved amino acids within the tyrosine kinase domain of the c-Kit receptor resulting in the disruption of the biochemical activity of

TABLE 1.4.1A: The phenotype of a selection of *W* mutant mice

W mutation	Pigmentation	Haemopoiesis	Gametogenesis	Viability	References
W/W		severe anaemia		lethal	Little (1915)
W/+	spotting	no anaemia	fertile	viable	
W^v/W^v	black-eyed, white	macrocytic anaemia	sterile	viable	Little & Cloudman (1937)
$W^v/+$	diluted coat; ventral spot	mild anaemia	fertile	viable	
W^{37}/W^{37}		severe anaemia		lethal	Geissler *et al.*, (1981)
$W^{37}/+$	mottled coat	no anaemia	fertile	viable	
W^{41}/W^{41}	mottled coat	severe anaemia	fertile	viable	Geissler *et al.* (1981)
$W^{41}/+$	diluted coat; ventral spot	mild anaemia	fertile	viable	
W^{42}/W^{42}		severe anaemia		lethal	Geissler *et al.* (1981)
$W^{42}/+$	black-eyed, white	macrocytic anaemia	fertile	viable	
W^{44}/W^{44}	small belly spots	no anaemia	sterile	viable	Geissler *et al.* (1981)
$W^{44}/+$	small belly spots	no anaemia	fertile	viable	
W^{57}/W^{57}	white patches	mild anaemia	fertile	viable	Reith *et al.* (1990)
$W^{57}/+$	white spot	no anaemia	fertile	viable	
W^{19H}/W^{19H}				lethal	Lyon *et al.* (1984)
$W^{19H}/+$	white spotting	no anaemia	fertile	viable	
W^{sh}/W^{sh}	black-eyed, white	no anaemia but mast cell def.	fertile	viable	Lyon and Glenister (1982)
$W^{sh}/+$	sash of white		fertile	viable	

the receptor (see table 1.4.1B). Mutations that abolish kinase activity due to deletion (*W* and W^{19H}) (Hayashi *et al.*, 1991; Chabot *et al.*, 1988) or point mutation (W^{37} and W^{42}) resulting in loss-of-function or "null" mutations (Nocka *et al.*, 1990b; Reith *et al.*, 1990; Tan *et al.*, 1990) are lethal in the homozygous state. In contrast, mutations which have residual kinase activity (W^{v} and W^{41}) are viable as homozygotes (Nocka *et al.*, 1990b; Reith *et al.*, 1990). Certain mutations that cause severe effects in the heterozygous state exhibit strong "dominant negative" effects (Herskowitz 1987). For example W^{v}, W^{41}, W^{42} which comprise of mutations at distinct sites in the region encoding the kinase domain of the *c-kit* gene, result in the production of impaired receptors with reduced or no intrinsic kinase activity but are nevertheless expressed on the cell surface (Reith *et al.*, 1990; Tan *et al.*, 1990). In heterozygotes, dimerization between wild type and mutant c-Kit receptors can occur. The mutant receptor therefore impairs the function of the wild type receptor by preventing transduction of the normal ligand-induced signal, in most cases preventing transphosphorylation of the wild type receptor within the dimer complex. Those mutations that result in lack (*W*) or reduced levels (W^{37}) of protein being expressed at the surface have less severe defects in heterozygotes (Nocka *et al.*, 1990b). The *W* mutant gene possesses a single base substitution (GT-AT) at the 5' splice donor site of the exon which encodes the transmembrane domain. This causes improper mRNA processing resulting in exon skipping producing a truncated product that is not expressed on the cell surface (Hayashi *et al.*, 1991) resulting in a 50% reduction in the number of functional receptors expressed at the surface in heterozygotes. W^{44} and W^{57} mutations produce reduced kinase activity which is associated with the reduced levels of mRNA and protein expressed in these mice. W^{44} was found to have a rearrangement within the c-*kit* gene whereas no gross rearrangement was found within the c-*kit* coding region in W^{57} mice (Geissler *et al.*, 1988; Reith *et al.*, 1990). Similarly, W^{sh} mutant mice express reduced levels of c-*kit* mRNA and protein in skin mast cells in an age-dependent manner resulting in a mast cell deficiency in the puppies of W^{sh} homozygotes but not in the embryos (Yamazaki *et al.*, 1994). As with W^{57} no alteration in the c-*kit* coding region of W^{sh} was found however the detection of a genomic rearrangement 50-100 kbp upstream of the c-*kit* coding region was revealed (Duttlinger *et al.*, 1993). Reduced c-Kit

TABLE 1.4.1B: *W* Mutations

W mutant	Kinase activity	c-*kit* mutation	dominant negative*	Reference
W	-	deletion	-	Hayashi *et al.*, (1991)
$W^v(=W^{55})$	±	$thr^{660}\rightarrow met$	+	Nocka *et al.*, (1990)
W37	-	$glu^{582}\rightarrow lys$	±	Nocka *et al.*, and Reith *et al.*, (1990)
W41	+	$val^{831}\rightarrow met$	+	Nocka *et al.*, and Reith *et al.*, (1990)
W42	-	$asp^{790}\rightarrow asn$	+	Tan *et al.*, (1990)
W44	+	insertion	-	Geissler *et al.*, (1988)
W57	+	regulatory	-	Reith *et al.*, (1990)
W19H	-	gene deleted	-	Geissler *et al.*, 1988
Wsh	+	regulatory	-	Duttlinger *et al.*, 1993

* dominant negative in the heterozygous state

protein and mRNA associated with the W^{57} and W^{sh} mutations are a consequence of disruption in regulation of c-*kit* transcription (Reith *et al.*, 1990; Duttlinger *et al.*, 1993; Dr. A. Bernstein, personal communication).

There are several aspects of the function of the c-Kit receptor that might be affected by W mutations, for example ligand binding, tyrosine kinase activity, receptor autophosphorylation, association of c-Kit with certain signal molecules, ligand induced internalisation, and receptor downregulation. The different c-Kit mutations were shown to differentially affect coat colour, anaemia, mast cell deficiency and fertility with different severities, demonstrating that different biochemical events triggered by the activation of the c-Kit receptor are of importance in these processes. Several ligand-induced c-Kit responses have been shown to be dependent or independent of certain cytoplasmic proteins that associate with the c-Kit receptor after activation. A few of those discovered thus far which are necessary for chemotaxis (migration), proliferation and adhesion have been described in section 1.7.3. Similar mechanisms may explain why a particular c-Kit mutation can for example have a more dramatic affect on anaemia than on fertility. The level of receptor expression has also been shown to be of importance in determining the biological outcome of a cell (for example proliferation versus differentiation described in detail in section 1.8.2) and this may also explain the phenotypes seen in those mice with reduced levels of functional protein in the heterozygous state (eg W/+ mice).

In humans a similar phenotype to that displayed by *W* mutant mice is also seen in patients with the autosomal dominant genetic disorder of piebaldism. The phenotype of these patients is characterised by patches of hair and skin which lack pigmentation, due to the absence of melanocytes in these areas. In contrast to the mutant mice, fertility and anaemia do not appear to be associated with the piebald trait (Fleischman *et al.*, 1991; Giebel & Spritz, 1991 and references therein; Ward *et al.*, 1995). The defect seen in these patients is also due to deletions or point mutations located within the c-*kit* gene on the long arm of chromosome 4, demonstrating piebaldism as the human homologue to the dominant white spotting of the mouse (Fleischman *et al.*, 1991; Giebel & Spritz, 1991; Ezoe *et al.*, 1995 and references therein). In some cases a deletion of the closely linked α*PDGFR* gene is also found

(Fleischman *et al.*, 1991). It is interesting to note that the W^{19H} mutant mouse has a complete deletion of the c-*kit* and α*PDGF* receptor genes which are located less than 650 kb apart on chomosome 5 (Smith, *et al.*, 1991). Deletion of part of the αPDGFR has been associated with the phenotype of *Patch* mice (*Ph*) which also display defects in pigmentation (white spotting) (Silvers 1979; Pawson & Bernstein, 1990). Another mouse with defects in pigmentation is the *rump-white (Rw)* mutant which may be genetically linked to both *W* and *Ph* (Giebel & Spritz, 1991).

1.5 The ligand to the c-Kit receptor: The Link between *W* and *Sl* mutant mice

Another set of mice displaying a similar phenotype to the *W* mutant mice are the *Steel* (*Sl*) mice, which have defects associated with the *Sl* locus localised on murine chromosome 10 (Sarvella *et al.*, 1956). Early experiments involving the analysis of the defects within the haemopoietic compartment revealed that the *W* mutant mice had a defect that was intrinsic to their stem cells now attributed to mutations within c-*kit*. In contrast, the *Sl* mutant mice had a defect within the microenvironment required to support growth of primitive stem cells (Russell 1979; Silvers 1979). These conclusions were drawn from transplantation experiments in which the injection of normal (+/+) bone marrow, as a source of stem cells, into unirradiated W/W^v mice resulted in the cure of their macrocytic anaemia and the production of spleen colony forming cells (CFU-S) (Russell *et al.*, 1959, Russell & Bernstein, 1968; McCulloch *et al.*, 1964). The injection of +/+ bone marrow cells into Sl/Sl^d mice could not cure their anaemia or produce CFU-S, suggesting that the stem cells within these mice were normal and that the microenvironment supporting the stem cells was impaired (McCulloch *et al.*, 1965; Altus *et al.*, 1971). These results were reproduced *in vitro* by Dexter & Moore (1977) using long-term bone marrow cultures (LTBMC), in which W/W^v stromal cells were able to support bone marrow cells of Sl/Sl^d mice but Sl/Sl^d stroma could not support W/W^v bone marrow cells.

Similarly, Kitamura and colleagues demonstrated that transplantation of normal bone marrow cells into W/W^v mice cured them of their mast cell deficiency. However, transplantation into Sl/Sl^d mice showed that these mice were also defective in the

microenvironment required to support mast cells (Kitamura *et al.*, 1978, 1979). +/+ mast cells could be derived from primitive stem cell progenitors when cultured in medium supplemented with pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM). It was later discovered that the growth factors supporting the growth of these cells were interleukin (IL)-3 and/or IL-4. +/+ mast cells could also be supported by cell-cell contact with fibroblast feeder layers (Levi-Schaffer *et al.*, 1986), in a similar way to the bone marrow cells supported by stromal layers in the absence of exogenous factors in LTBMC (Dexter & Moore, 1977). The support of mast cells in these co-culture systems was not due to IL-3 or IL-4 production by the feeder layer demonstrating that another mast cell growth factor was involved Fujita *et al.*, (1988,1989). Since this system consisted of two homogeneous populations it provided a means to analyse the cell-cell interaction between the mast cells and the NIH/3T3 cells.

The surface expression of c-Kit by mast cells was shown to be necessary for their attachment to fibroblasts, since inhibition could be achieved by the α-c-Kit monoclonal antibody (mAb), ACK2 and *W/W* mast cells which lacked c-Kit surface expression did not attach (Adachi *et al.*, 1992). Mast cells with defective c-Kit receptors from W^{37}/W^{37}, W^{41}/W^{41}, W^{44}/W^{44}, W^{57}/W^{57} and W^{v}/W^{v} mice were able to bind to the feeder layers but were compromised in their ability to grow, demonstrating that functional c-Kit receptors were required for optimal growth. Less dramatic effects were seen with mast cells obtained from heterozygous mice (Nocka *et al.*, 1990b; Reith *et al.*, 1990; Adachi *et al* 1992). These results reflected the severity of the defects seen *in vivo* in the *W* mutant mice (see table 1.4.1A & B) and suggested that the ligand for c-*kit* was expressed by the fibroblast cells.

1.5.1 Isolation and Characterisation of *Steel* factor (SLF)

It wasn't until the discovery of the c-Kit ligand in 1990 that Russell's theory that the complementary phenotypes of the *W* and *Sl* mutant mice were a result of the disruption between the interaction of a growth factor receptor and its ligand was proven to be correct. Nocka *et al.*, (1990a) purified the Kit ligand (KL) from conditioned medium collected from Balb/c 3T3 +/+ fibroblasts. The gene encoding the ligand for the c-Kit receptor was found to map to the *Steel* (*Sl*) locus on mouse chromosome 10 by many groups (Copeland *et al.*, 1990;

Huang *et al.*, 1990; Zsebo *et al.*, 1990a) and on human chromosome 12 (Anderson *et al.*, 1991). The ligand was isolated from many sources and given several names which include: Kit ligand (KL) (Nocka *et al.*, 1990a) isolated from Balb/c 3T3 fibroblasts, mast cell growth factor (MGF) purified from a murine stromal cell line, LDA11, (Anderson *et al.*, 1990; Boswell *et al.*, 1990; Williams, *et al.*, 1990), stem cell factor (SCF) purified from Buffalo rat liver-conditioned medium (Zsebo *et al.*, 1990b) and *Steel* factor (SLF) (Williams, *et al.*, 1992), the latter which will be used throughout the remainder of this thesis.

SLF cDNA encodes a transmembrane polypeptide with a secretion signal peptide of 25 amino acids, a 189 amino acid extracellular domain, a 23 amino acid hydrophobic transmembrane domain and a 36 aa intracellular domain (Anderson *et al.*, 1990; Huang *et al.*, 1990; Martin *et al.*, 1990). Two forms of *SLF* mRNA have been described in both human and murine systems. The two transcripts are produced due to alternative splicing, resulting in a 248 amino acid protein (SLF^{248}) or one which is 28 amino acids smaller (SLF^{220}) lacking exon 6 (Anderson *et al.*, 1991; Flanagan *et al.*, 1991a; Huang *et al.*, 1992; Toksoz *et al.*, 1992). Within this exon lies the recognition site for a serine protease involved in proteolytic cleavage (Martin *et al.*, 1990; Lu *et al.*, 1991; Pandiella *et al.*, 1992) of SLF^{248} releasing a 164-165 aa soluble SLF protein from the cell membrane (Nocka *et al.*, 1990, Williams *et al.*, 1990, Zsebo *et al.*,1990a). Proteolytic cleavage of SLF^{220} also occurs in mice, even though it has lost this proteolytic cleavage site (Huang *et al.*, 1992; Pandiella *et al.*, 1992; Majumdar *et al.*, 1994). This is the result of utilisation of a secondary or alternate proteolytic cleavage site, encoded in exon 7, which is present in both murine SLF^{248} and SLF^{220}. Human SLF does not possess this site due to species divergence within the sequence in this area and therefore human SLF^{220} remains associated with the membrane (Majumder *et al.*, 1994). Murine SLF^{220} cleavage has been shown to involve a serine protease of different specificity to that involved in the cleavage of SLF^{248}. Nevertheless, SLF^{248} cleavage is more efficient than that of SLF^{220} due to the predominance of this transcript in various tissues and the variance in the level of expression suggests that the SLF isoforms are regulated in a tissue-specific manner (Huang *et al.*, 1992). The ligands to the *flk2/flt3* and CSF-1 receptors

also exist as different isoforms due to tissue specific splicing and proteolytic cleavage (Ladner *et al.*, 1987; Lyman *et al.*, 1995)

1.5.2 *Sl* Mutant Mice

As with the *W* mutant mice, many spontaneous *Steel* (*Sl*) mutant mice also exist (Russell *et al.*, 1966; Silvers 1979). The mutant *Sl* alleles that have the most dramatic effects, generally resulting in death *in utero* or shortly after birth, are due to deletions that produce complete loss of SLF function. Such alleles include *Sl* (Sarvella *et al.*, 1956) as well as Sl^{J}, Sl^{gb}, Sl^{10H}, Sl^{12H} and Sl^{18H} (Russell 1979; Silvers 1979). In contrast, mice homozygous for the *Steel-Dickie* (Sl^{d}) allele are viable, despite exhibiting moderately severe macrocytic anaemia, a complete lack of skin pigmentation, a profound mast cell deficiency and sterility in both sexes (Kitamura *et al.*, 1979; Russell 1979; Silvers 1979). The Sl^{d} allele encodes an abundant but truncated SLF transcript that encodes almost all of the extracellular domain of SLF but lacks coding sequences for the transmembrane and intracellular domains of the wild type protein. Nine amino acids of the extracellular domain are missing and are replaced by 3 sense codons followed by a stop codon resulting in the production of a biologically active secreted form of SLF but no membrane-bound isoform (Zsebo *et al.*, 1990a; Brannan *et al.*, 1991; Flanagan *et al.*, 1991). The impairment of SLF function associated with the deletion of the transmembrane and intracellular domains is remarkably severe, since Sl/Sl^{d} and Sl^{d}/Sl^{d} mice are almost as heavily impaired as *Sl/Sl* mice which have a complete deletion of the SLF gene (Russell 1979; Silvers 1979). This suggests that soluble SLF produced by the Sl^{d} allele fails to fulfil the normal biological functions of this growth factor and defines the importance of membrane-bound SLF in the processes affected in these mice (discussed further in section 1.5.3).

Several other *Sl* mutant mice have been analysed and appear to shed light on the importance of SLF in gametogenesis. Homozygotes for the *Steel-panda* (Sl^{pan}), *Steel-contrasted* (Sl^{con}) alleles produce female sterility, whereas Sl^{17H} homozygotes produce sterility in the male only (reviewed by Bedell *et al.*, 1995). Sl^{con} and Sl^{pan} mutations lead to tissue specific effects on SLF mRNA expression. A decrease in SLF

expression causes sterility in Sl^{pan} and Sl^{con} homozygous females by affecting the initiation and maintenance of ovarian follicle development. SLF is ectopically expressed at high levels in some tissues of Sl^{con} mice and causes abnormal accumulation of mast cells and melanocytes (Bedell *et al.*, 1995). The Sl^{17H} mutation results in an SLF product which is shortened by 8 aa at the cytoplasmic tail. Analysis of germ cell development in Sl^{17H}/Sl^{17H} mice indicates that SLF has distinct roles in both embryogenic and adult development. During embryogenesis in male and female germ cell development SLF appears to have equivalent functions but in male adults performs sex-specific functions that require the wild-type cytoplasmic tail (Brannan *et al.*, 1992). It has been postulated that the cytoplasmic tail of membrane-bound SLF may itself be able to transmit a signal (Flanagan *et al.*, 1991). Although no evidence supports this hypothesis as yet, this may explain the phenotype displayed by male Sl^{17H}/Sl^{17H} mice. Sl^{con}, Sl^{pan} *and* Sl^{17H} mutant mice all display mild anaemia and exhibit partial coat pigmentation, suggesting that the mutations produce only limited impairment of SLF function in comparison to the *Sl* and Sl^{d} mutations.

Another set of mutant mice with a disruption in the production of a growth factor are the *op/op* mice. Mice homozygous for mutations at the *op* locus (chromosome 3) have a marked deficiency in macrophages and osteoclasts, and hence are severely osteopetrotic. *Op/op* fibroblasts are deficient in CSF-1 production. The CSF-1 gene in *op/op* mice has a single base pair insertion in the coding region, resulting in a stop codon 21 bp downstream of the translation start site (Pawson & Bernstein, 1990 and references therein). Mice carrying mutations in the *microphthalmia* (*mi*) locus display a phenotype of a combined *W/Sl* and *op/op* mutant mouse having white coats, mast cell deficiency and oesteopetrosis. *Mi/mi* mast cells do not respond to SLF and are impaired in their attachment to feeder layers possibly due to a reduction in c-*kit* mRNA and protein detected in these cells. The *mi* locus has been identified to encode a novel member of the basic-helix-loop-helix-leucine zipper family (Hughes *et al.*, 1993) and may play a role in the regulation of c-*kit* expression (Ebi *et al.*, 1992; Isozaki *et al.*, 1994).

1.5.3 Differential roles of membrane-bound and soluble SLF

As discussed in section 1.5.2 the *Sl/Sl*^{d} mutant mice have demonstrated that membrane-bound SLF fulfils a function that is not compensated for by soluble SLF. The cells typically affected by mutations at the *Sl* and *W* loci undergo migrations during normal development: primordial germ cells migrate from the yolk sac to the genital ridges, haemopoietic cells from the yolk sac to the embryonic liver and then to the spleen and bone marrow, and melanocyte precursors from the neural crest to the skin and the hair follicles. The defects in the *Sl* and *W* mutants become apparent at about the time these migrations would normally occur, so it is an attractive possibility that the membrane-bound SLF/c-Kit interaction may have some special role in guiding cells during migration and homing (Russell 1979; Silvers 1979). This idea has been supported by *in situ* hybridisation analysis of c-*kit* and SLF transcripts in murine embryonic tissues which display a correlation between SLF/c-Kit interactions and the sites of migration and homing of germ cells, melanocyte precursors, and haemopoietic cells (Keshet *et al.*, 1991). However, soluble SLF may also play a role in migration by setting up concentration gradients allowing chemotaxis of c-Kit expressing cells (Blume-Jensen *et al.*, 1991; Meininger *et al.*, 1992). This could explain how high concentrations of recombinant soluble SLF (30 μg/kg/day) injected into *Sl/Sl*^{d} mice partially reversed their macrocytic anaemia and recruited mast cells to the site of injection (Zsebo *et al.*, 1990 a).

The adhesion between c-Kit expressing cells and membrane-bound SLF may not be solely due to the c-Kit/membrane-bound SLF interaction, but may involve other extracellular matrix proteins. It has been demonstrated that although both +/+ and *W/W*^{v} mast cells can bind to cell transfectants expressing the membrane-bound form of SLF, only +/+ cells are able to stimulate adhesion to the fibronectin receptor, VLA-5. This is achieved through SLF induced tyrosine kinase activation of the wild type c-Kit receptor, resulting in an "inside out" signalling of the fibronectin receptor. This is not accomplished by the *W/W*^{v} c-Kit receptor since its signalling is impaired (Dastych & Metcalfe, 1994; Kinashi *et al.*, 1994). Similarly, purified CD34+ human bone marrow cell populations, which contain primitive

stem/progenitor cells expressing c-Kit, and CD34+/c-Kit positive cell lines, M07e and TF-1 show enhanced adhesiveness to fibronectin due to upregulation of VLA-5 function upon SLF-stimulation (Lévesque *et al.*, 1995). Fibronectin has been shown to be involved in migration pathways and is widely expressed. The localised expression of SLF along the migratory pathways of the cell types affected by *W* and *Sl* mutants may serve to direct the migration of c-Kit expressing cells to their destinations. Other extracellular matrix proteins such as hemonectin (Anklesaria *et al.*, 1991) and thrombospondin (Long *et al.*, 1992) may also be involved in mediating SLF-stimulated adhesion.

It has recently been shown that the activation of c-Kit via membrane-bound SLF is able to induce more persistent tyrosine kinase activation of the receptor and a longer life span of the Kit protein compared to soluble SLF (Miyazawa *et al.*, 1995). The more persistent signalling may be due to prevention of internalisation of the Kit/membrane-bound SLF complex due to the association of SLF with the membrane. Soluble SLF, however, is able to downmodulate the c-Kit receptor through internalisation therefore acting as a turn-off switch for activated c-Kit kinase (see also section 1.7.4). The differential c-Kit activation produced by the SLF isoforms can explain why membrane-bound SLF is more effective in supporting stem cells and mast cells in co-culture systems than soluble SLF (Toksoz *et al.*, 1992; Fujita *et al.*, 1989) and why soluble SLF is relatively ineffective in Sl/Sl^d mice.

Another possible function assigned to membrane-bound SLF is its ability to provide high local concentrations of factor at specialised sites within the microenvironment. This could help to ensure proliferation and differentiation of cells only in the correct locations: for example haemopoietic progenitor cells in the spleen and bone marrow, mast cells in the skin, melanocytes in the skin and hair follicles and germ cells by Sertoli cells. Membrane-bound SLF may serve to "anchor" the cells within localised "niches" of these microenvironments facilitating the interaction of the cells with nearby molecules such as extracellular matrix proteins and other membrane-bound or locally produced cytokines which induce proliferation and/or differentiation (Gordon 1991; Lowry *et al.*, 1992). Proteolytic cleavage of membrane-bound SLF, as described in section 1.5.2, may release c-Kit expressing cells attached to feeder layers by this c-Kit/SLF interaction and allow them to migrate away.

Soluble SLF on its own has been shown to support cell survival and viability of haemopoietic stem cells, mast cells, melanocytes, primodial germ cells and natural killer (NK) precursor cells in certain culture systems without promoting proliferation (reviewed in Galli *et al.*, 1994). Recent data imply that SLF-induced survival of these cells may be due to rescue from cell apoptosis (Caceres-Cortes *et al.*, 1994; Carson *et al.*, 1994; Iemura *et al.*, 1994; Yee *et al.*, 1994b; Galli *et al.*, 1995). In general, SLF on its own is a relatively ineffective growth factor, however in the presence of other cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), erythropoietin (Epo), IL-3, IL-4, IL-6, IL-9 and gamma interferon (IFNγ) it demonstrates quite potent synergism. In combination with these cytokines SLF is able to promote and/or enhance colony formation of murine and human myeloid progenitors (ie. differentiation) (see section 1.6) and the proliferation of c-Kit expressing cells (Broxmeyer *et al.*, 1991b, c; Hendrie *et al.*, 1991; McNiece *et al.*, 1991a; Metcalf & Nicola, 1991). Mast cells are the only lineage which are able to survive, proliferate and differentiate in SLF alone (reviewed by Galli *et al.*, 1994). It has been demonstrated that under certain circumstances mast cells are able to produce other cytokines such as IL-3 and IL-4 upon SLF stimulation. Similarly SLF has induced the production of autocrine factors in other c-Kit expressing cell lines (Kiss *et al.*, 1993; Sasaki *et al.*, 1995) which may explain how these cells proliferate in the presence of exogenously added SLF alone (see also section 1.9.3.2).

1.6 c-Kit/SLF involvement in Haemopoiesis

Haemopoiesis is the process whereby mature blood cells, both myeloid and lymphoid, are derived in a hierarchal fashion from the pluripotent stem cell. This cell has the capacity to self-renew (ie produce an identical daughter cell) or differentiate into multipotential progenitor cells (CFU-GEMM), which in turn give rise to more lineage-restricted or committed progenitor cells (CFU-GM, BFU-E, BFU-MK) that are the precursors to various mature cell lineages. Haemopoiesis is regulated by a complicated network of growth factors, cytokines and extracellular matrix molecules produced by the stroma. Many assays have been developed in order to try and determine the phenotype of the most primitive stem cell able to

reconstitute haemopoiesis (for example the LTBMC and delta assay) (reviewed by Gordon 1993). The *W* and *Sl* mutant mice demonstrated the importance of the c-Kit/SLF interaction and as a result investigators have tried to identify the most primitive cell expressing c-Kit and assay its potential.

Early characterisation of W/W^v and Sl/Sl^d mutant mice demonstrated that they had reduced numbers of pluripotent stem cells, as assayed by the production of colony forming units in the spleen (CFU-S) (McCulloch *et al.*, 1964). It has been shown that both CFU-S and most of the colony forming cells (CFC) of the bone marrow express the c-Kit receptor. Only a small fraction of total murine and human bone marrow cells expresses c-Kit (~8% and 4%, respectively) (Ashman *et al.*, 1991; Ogawa *et al.*, 1991). In mice the cells able to reconstitute lethally irradiated mice are found in the c-Kit positive fraction (Ogawa *et al.*, 1991; Okada *et al.*, 1991). Injection of an antagonistic mAb, ACK2, to the murine c-Kit receptor into mice eliminated the production of all day 8 CFU-S, but not all day 12 CFU-S (Ogawa *et al.*, 1991; Okada *et al.*, 1991). These results suggest that, although c-Kit is expressed on a very early cell, that a more primitive cell that does not express c-Kit may exist. Hirabiyashi *et al.*, (1991) have further demonstrated this since secondary transplant of the c-Kit negative fraction of bone marrow gave rise to CFU-S in secondary recipients at day 14. These results may explain why *Sl/Sl* fetuses have reduced but detectable haemopoietic stem cells yet do not produce SLF able to interact with progenitor cells expressing c-Kit (Ikuta *et al.*, 1992). Similarly, human long term initiating cells have been shown to survive in the absence of SLF (Sutherland *et al.*, 1993) and the production of BFU-E from the CD34+Kit- fraction (Gunji *et al.*, 1993; Simmons *et al.*, 1994) and the inability to block BFU-E production in the presence of an antagonistic α-c-Kit mAb, SR-1 (Broudy *et al.*, 1992) may provide preliminary evidence for such a cell existing. It is therefore possible that the c-Kit negative cells may be supported by other receptor/ligand interactions now known to also play a role in haemopoiesis; for example IL-11 (Neben *et al.*, 1994; Quesniaux 1994; de Haan *et al.*, 1995) and the flk2/flt3 receptor and its ligand (Lyman *et al.*, 1993; Hirayama *et al.*, 1995; Hudak *et al.*, 1995), or other unidentified molecules. Nevertheless, *W/Sl* mutant mice demonstrate that the c-Kit/SLF interaction is vital for the progression of normal haemopoiesis.

As committed progenitors differentiate and become more lineage restricted the level of expression of c-Kit diminishes, except for mast cells which still retain high levels of the c-Kit receptor (Mayrhofer *et al.*, 1987). SLF displays its greatest potency in the presence of other cytokines in order to produce more committed progenitors. SLF synergises with IL-3 or GM-CSF to enhance Epo-dependent CFU-GEMM colony formation, with GM-CSF to enhance the production of CFU-GM, with erythropoietin (Epo) to enhance the production of BFU-E and with GM-CSF or IL-3 or IL-6 to enhance BFU-Mk (Bernstein *et al.*, 1991; Briddell *et al.*, 1991; Broxmeyer *et al.*, 1991a, c; Carow *et al.*, 1991; Dai *et al.*, 1991; McNiece *et al.*, 1991a; Metcalf & Nicola, 1991; Migliaccio *et al.*, 1991). Thus a main function of SLF is to enhance the proliferative capacity of progenitor cells responding to other cytokines. Therefore SLF may be an earlier acting factor than IL-3 or GM-CSF (Carow *et al.*, 1991; Broxmeyer *et al.*, 1991a, c). However, it is the factor that SLF is synergising with which determines the phenotype of the colony. SLF is also able to reduce the concentration of the second cytokine required to produce optimal colony formation (McNiece *et al.*, 1991; Metcalf & Nicola, 1991). Apart from the cytokines already mentioned, SLF acts in combination with: IFNγ to enhance BFU-Mk (Shiohara *et al.*, 1993), IL-7 to enhance the proliferation of pre-B cells (McNiece *et al.*, 1991b; Ogawa *et al.*, 1991; Billips *et al.*, 1992), IL-2 in the development of natural killer (NK) cells (Matos *et al.*, 1993), IL-4 and IL-10 for growth and maturation of mast cell progenitors (Rennick *et al.*, 1995) and IL-11 to enhance the proliferation and expansion of murine progenitor cells (Tsuji *et al.*, 1992; Neben *et al.*, 1994). The role of SLF/c-Kit in melanocyte and germ cell development has been reviewed by Galli *et al.*, (1994).

1.7 Biochemical analysis of the c-Kit receptor

1.7.1: Ligand Binding Domain

As predicted by the specificity, the ligand binding domains of related receptors belonging to the same RTK family are characterised by a low degree of sequence conservation. However, members of the ErbB receptor family (ie RTK type I) are able to bind a number of related ligands including epidermal growth factor (EGF), transforming growth

factor (TGF)-α, amphiregulin and proposed ligands of the HER-2/*neu* (ErbB2) receptor (reviewed by Lemmon and Schlessinger, 1994). A receptor may also bind the cognate ligand of a different species, for example human CSF-1 binds to murine CSF-1R although murine CSF-1 cannot bind to the human CSF-1R (Das *et al.*, 1982). The reverse is observed with the c-Kit receptor, murine or rat SLF is able to bind to both murine and human c-Kit receptors, whereas the binding of human SLF to the murine c-Kit receptor is almost non-existent (Martin *et al.*, 1990; Lev *et al.*, 1993).

The extracellular domain of the c-Kit receptor consists of five distinct Ig-like domains. In order to determine the ligand binding domain of the c-Kit receptor Lev *et al.* (1993) exploited its species specificity by producing chimeric c-Kit receptors consisting of murine and human c-Kit domains. Using the mouse receptor as the backbone and substituting human domains into it the binding site of human SLF was localised. The production of interspecies chimeric extracellular domains of growth factor receptors to determine ligand binding sites had been previously employed with the EGF receptor combining human and chicken domains, isolating the ligand binding site to the sequences (domains I and III) that connect the two cysteine-rich repeat domains (Lax *et al.*, 1989, 1991). As well as the chimeric receptor model, epitope mapping using a number of mAb to the extracellular domain of c-Kit, combined with analysis of their abilities to block ligand binding were used to localise the SLF binding site. Soluble ectodomains of the c-Kit receptor were also employed to determine the minimal requirements of the extracellular domain for ligand binding (Blechman *et al.*, 1993a, b).

The ligand binding domain was confined to the three N-terminal Ig-like domains with the second domain conferring high-affinity binding of human SLF (Lev *et al.*, 1993). Domains I and III, however assisted domain II in its binding of ligand and a soluble protein possessing only the first two domains showed reduced affinity of SLF binding compared to one possessing all three domains. High-affinity binding involving the three N-terminal domains may result from domain III folding over the binding cleft in domain II and thereby inhibiting ligand dissociation. The ability of non-contiguous domains to stabilise ligand binding is also seen with the EGFR (as described above) and insulin receptors (Schumacher

et al., 1991). Ligand competitive c-Kit mAb (K44 and K57; Blechman *et al.*, 1993a) were also found to bind to domain II which confirmed that this domain contains the binding site for human SLF. This is also in accordance with the localisation of the binding of PDGF-AA to the second domain of the αPDGFR (Heidaran *et al.*, 1992). Similar studies were performed to determine the ligand binding site of rodent SLF to the murine c-Kit receptor, using the human receptor as the backbone and substituting in murine c-Kit domains. The SLF binding site of the murine receptor lies mainly in domain III with some contribution from domain II. Therefore the binding domains of human and rodent SLF appear to be distinct but overlap to some extent.

1.7.2: Dimerization

Ligand binding to the extracellular domain of RTK has been shown to trigger an intermolecular mechanism involving oligomerization of the receptor molecules, resulting in the activation of their intrinsic protein tyrosine kinase and autophosphorylation (Yarden *et al.*, 1987b, c; Bishayee *et al.*, 1989; Honegger *et al.*, 1990; Li *et al.*, 1991) and reviewed in Ullrich *et al.*, 1990; Heldin 1995). The mechanism of ligand induced dimerization is still under investigation and many models have been proposed. For the EGFR where the ligand is a monomer, it has been proposed that ligand binding induces conformational changes within the ligand bound receptors bringing them together to form a dimeric complex (Greenfield *et al.*, 1989). However, it has recently been demonstrated that EGF is multivalent and can simultaneously bind by distinct sites to two EGFR molecules (Lemmon and Schlessinger, 1994).

However the ligands of the type III RTK, including SLF, exist as dimers and it is still controversial whether the dimeric ligand brings about dimerization of neighbouring receptors as a result of each ligand subunit binding a single receptor or whether the ligand binds to one receptor and then induces conformational changes leading to receptor dimerization. Dimerization of growth factor receptors enhances ligand binding affinity, activates protein tyrosine kinase activity and autophosphorylation of the receptor (Boni-Schnetzler *et al.*, 1987; Yarden *et al.*, 1987b, c; Bishayee *et al.*, 1989; Heldin *et al.*, 1989; Williams *et al.*, 1989;

Ben-Levy *et al.*, 1992; Spivak-Kroizman *et al.*, 1992; Vassbotn *et al.*, 1993; Lemmon and Schlessinger, 1994; Schlessinger 1995).

Regarding the c-Kit receptor, Blume-Jensen *et al.*, (1991) demonstrated that the dimeric SLF molecule was responsible for inducing receptor dimerization of neighbouring receptors. This was concluded from results demonstrating that at excess concentrations SLF binds monovalently resulting in a decrease in dimeric c-Kit receptors. Similar events were shown to occur with the PDGFR (Heldin *et al.*, 1989) However, Lev *et al.*, (1992b, c) provided evidence that this model of ligand induced dimerization may not be totally correct. They failed to see inhibition of c-Kit dimer formation in the presence of excess ligand. Furthermore incubating purified human (deletion mutant lacking COOH-terminal 42 aa) and murine c-Kit receptors in the presence of human SLF which is species-selective was able to induce cross-species receptor heterodimerization (Lev *et al.*, 1992 b). These results implied that monovalent binding of human SLF was sufficient to induce c-Kit dimerization and activate the receptor complex. The model they proposed was that the SLF dimer binds to one receptor which induces a conformational change resulting in the "opening" of an internal dimerization site. This results in the association with another receptor molecule (unstable dimer), which can then bind the other arm of the ligand dimer "opening" the dimerization site in this receptor molecule forming a stable high-affinity ligand binding dimer complex. Trimers and tetramers did not form due to the monovalence of the putative dimerization site (model reviewed in Lev *et al.*, 1992b). This model demonstrates that not only ligand binding induces receptor dimerization, but that an intrinsic interaction occurs between the receptor-receptor complex.

The putative dimerization site of c-Kit has recently been localised to domain IV of the extracellular domain using deletion mutants and a mAb able to inhibit dimerization (Blechman *et al.*, 1995). These data show that the extracellular domain of c-Kit is sufficient to induce dimerization which is in contrast with the EGFR (Günther *et al.*, 1990) and the HER-2/*neu* receptor (Weiner *et al.*, 1989). In the latter case dimerization is attributed to the transmembrane domain (although the extracellular domain stabilises dimerization) as an oncogenic form of the HER-2/*neu* receptor which possess a single point mutation

$Val^{664} \rightarrow Glu^{664}$ within this domain is constitutively dimeric and has tyrosine kinase activity (Bargmann *et al.*, 1988; Weiner *et al.*, 1989; Qian *et al.*, 1995). The localisation of the c-Kit dimerization site to domain IV coincides with several activating mutations within this domain of the CSF-1 receptor, which result in receptor dimerization (van Daalen-Wetters *et al.*, 1992; Carlberg *et al.*, 1994). The only dimerization site thus far confirmed by crystallography is that of the growth hormone receptor (de Vos *et al.*, 1992).

1.7.3: Signalling

As discussed above, dimerization is a prerequisite for the activation of unaltered RTKs and the subsequent recruitment of cytoplasmic molecules involved in signal transduction results in a cellular response. Following dimerization the receptor molecules become phosphorylated on tyrosine residues within the cytoplasmic domain. This process is achieved by one receptor trans-phosphosphorylating the other within the dimer (reviewed in Ullrich and Schlessinger, 1990 and Heldin, 1995). The tyrosine residues that become phosphorylated reside within the tyrosine kinase domain, juxtamembrane domain, kinase insert and carboxyl tail. The phosphorylated tyrosine residues in c-Kit have not been systematically mapped as has for the βPDGFR, which contains 9 phosphorylated tyrosine residues (see Claesson-Welsh 1994, figure 2), of these only one exists in the tyrosine kinase domain. This residue is conserved between RTKs (Y_{857} of the human βPDGFR (Claesson-Welsh, 1994); Y_{807}, Y_{809} of the murine and human CSF-1R, respectively (reviewed in van der Geer & Hunter, 1993); Y_{821}, Y_{823} in murine and human c-Kit receptors, respectively (Serve *et al.*, 1995)). Its autophosphorylation in type III RTKs increases kinase activity and precedes phosphorylation of other sites in the receptor or substrates (Hanks *et al.*, 1991). Substitution of Y_{821} in the murine c-Kit receptor by a phenylalanine residue impaired SLF-induced proliferation and survival of bone marrow derived mast cells (BMMC) but not their adhesion to fibronectin. The downstream events affected by this mutation at present are not known but were independent of phosphatidylinositol 3'-kinase (PI 3'-K), $p21^{ras}$ and mitogen-activated protein kinase (MAPK) activation and did not affect induction of the early response gene c-*fos* and c-*jun* (Serve *et al.*, 1995). Similar mutations of the homologous residues in the CSF-1R

and βPDGFR impair mitogenic signalling (reviewed in van der Geer and Hunter, 1993; Claesson-Welsh 1994). Phosphorylation of this conserved residue does not occur within the EGFR; its autophosphorylation sites are clustered at the carboxyl tail (Yarden and Ullrich, 1988). Autophosphorylation of tyrosine residues which lie outside of the kinase domain function as docking sites for downstream signal transduction molecules which contain *src*-homolgy 2 (SH2) domains (for review of the signalling molecules associated with the PDGFR see Claesson-Welsh, 1994). The SH2 domains consist of about 100 amino acids folded in such a way that a binding pocket for a phosphorylated tyrosine residue and the immediately surrounding amino acids, (which gives the interaction specificity) is formed (reviewed in Cantley *et al* 1991; Pawson & Schlessinger, 1993; Cohen *et al.*, 1995).

Many groups have shown that the SLF-induced phosphorylation of the c-Kit receptor results in the binding of the p85 subunit of phosphatidylinositol 3'-kinase (PI 3'-K) in a number of different cell types (Lev *et al.*, 1991, 1992a; Reith *et al.*, 1991a; Rottapel *et al.*, 1991; Shearman *et al.*, 1993; Blume-Jensen *et al.*, 1994; Serve *et al.*, 1994). PI 3'-K is a heterodimer composed of an 85 kd SH2-containing regulatory and a 110 kd catalytic subunit; binding of p85 to phosphorylated tyrosine activates PI 3'-kinase activity. Using mutants lacking part of the interkinase domain Lev *et al.* (1992a) localised this as the binding region for p85 and recently Y_{719} of the murine c-Kit receptor was shown to be the critical tyrosine residue involved (Serve *et al.*, 1994) and the corresponding residue in the human receptor is Y_{721}(Shearman *et al.*, 1993). The phosphorylated tyrosine residues associated with the binding of p85, within the interkinase domain, to the human βPDGFR (Y_{740} and Y_{751}) (Claesson-Welsh 1994 and references therein) and CSF-1R (Y_{721}) (Reedijik *et al.*, 1992) were determined from mutagenesis and phosphopeptide competition studies and are homologous to Y_{719}/Y_{721} (murine/human) of the c-Kit receptor. Mutation of this residue in the murine c-Kit receptor abolished PI 3'-K activation and diminished c-*fos* and c-*jun* induction, which correlated with the impairment of BMMC to adhere to fibronectin in the presence of SLF (see also section 1.5.3) but had a minimal affect on cell proliferation and survival (Serve *et al.*, 1995). Stimulation of the c-Kit receptor not only results in p85 association but also phosphorylation of the subunit (Blume-Jensen *et al.*, 1994). Deletion of

the interkinase domain of the CSF-1R demonstrated that autocrine induced anchorage-independent growth of NIH/3T3 cells was independent of PI 3'-K association (Taylor *et al.*, 1989). The deletion/phenylalanine substitution of the two tyrosines involved in p85 binding to the αPDGFR did not affect mitogenic signalling nor ligand-mediated transformation however p85 was required for chemotactic responses (Heidaran *et al.*, 1991; Yu *et al.*, 1995). These data demonstrate that PI 3'-K is involved in the regulation of different cellular responses and transformation induced by the various RTKs (Kapeller & Cantley, 1994)

The c-Kit receptor has also been demonstrated to associate and weakly phosphorylate phosholipase C-γ1 (PLC-γ1) (Rottapel *et al.*, 1991; Lev *et al.*, 1991; Blume-Jensen *et al.*, 1994). Although Rottapel *et al.* (1991) showed PLC-γ1 association with c-Kit in murine mast cells, Koike *et al.*, (1993) demonstrated phospholipase D (PLD) rather than PLC-γ1 association in rat mast cells. Unlike PI 3'-K which was found to associate with all members of the type III RTKs, PLC-γ1 was unable to associate with CSF-1R (Downing *et al.*, 1989a). It is interesting to note that although the EGFR was also able to activate PLC-γ1-it was dependent on high EGFR expression (Wahl *et al.*, 1988; Margolis *et al.*, 1989). The binding of PLC-γ1 to the αPDGFR has recently been shown to be involved in mediating focus-formation of NIH/3T3 cells expressing a chimeric CSF-1/αPDGFR but is not required for anchorage-independent growth or chemotaxis (Yu *et al.*, 1995). It has recently been reported that PI 3'-K and PLC-γ1 compete for association to their binding sites on the c-Kit receptor with the former exhibiting higher affinity however these proteins bind simultaneously to the β PDGFR (Herbst *et al.*, 1995b).

Weak association of the Ras GTPase-activating protein (GAP) with c-Kit has been demonstrated in human embryonic kidney fibroblast cells, 293T (Herbst *et al.*, 1991), and haemopoietic cells (Duronio *et al.*, 1992) but no association was found in mast cells (Rottapel *et al.*, 1991). Weak association/phosphorylation with GAP was also shown to occur with the flk2 and CSF-1 receptors (Reedijk *et al.*, 1990; Dosil *et al.*, 1993) whereas GAP association/phosphorylation with the PDGFR was very strong (Kaplan *et al.*, 1990). SLF induces phosphorylation of a number of other substrates in a variety of cell backgrounds.

These include: Raf-1 kinase on serine and mitogen-activating protein kinase (MAPK) on threonine and tyrosine residues (Lev *et al.*, 1991; Funasaka *et al.*, 1992; Hallek *et al.*, 1992; Okuda *et al.*, 1992; Welham & Schrader, 1992; Blume-Jensen *et al.*, 1994), Tec kinase in M07e cells (Tang *et al.*, 1994), $p95^{vav}$ in M07e and TF-1 cells (Alai *et al.*, 1992), Grb2/Sem-5 (Blume-Jensen *et al.*, 1994), and phosphotyrosine phosphatase in melanocytes (Funasaka *et al.*, 1992) and haemopoietic cells (the haemopoietic phosphatase (HCP) or PTP1C) (Yi *et al.*, 1993), the latter which negatively regulates c-Kit signalling (Paulson *et al.*, 1996; Piao *et al.*, 1996).

Serine phosphorylation is also induced by SLF stimulation of the c-Kit receptor. In contrast to tyrosine phosphorylation which increases the kinase activity of the receptor, serine phosphorylation induced by protein kinase C (PKC) acts as a negative regulator of tyrosine autophosphorylation but does not affect ligand binding affinity (Blume-Jensen *et al.*, 1994) (see also section 1.7.4). Recently S_{741} and S_{746} located in the interkinase domain, S_{821} close to the major autophosphorylation site (Y_{823}) and S_{959} in the carboxyl terminus have been identified as serine phosphorylation sites in the human c-Kit receptor (Blume-Jensen *et al.*, 1995). PKC activation by c-Kit was shown to be required for SLF induced cell motility (chemotaxis/migration) but not proliferation (Blume-Jensen *et al.*, 1993). The EGFR is also negatively regulated by PKC induced phosphorylation of serine and threonine residues inhibiting both kinase activity and ligand binding affinity (Lin *et al.*, 1986) .

Several of the growth factor RTKs have been ectopically expressed in IL-3 and/or GM-CSF dependent murine haemopoietic cell lines resulting in the abrogation of dependency on these growth factors. Instead these cells are able to proliferate in response to the ligand corresponding to the introduced RTK which include the CSF-1R (Kato *et al.*, 1989; Dibb *et al.*, 1990; Pierce *et al.*, 1990; von Rüden *et al.*, 1991), EGFR (Pierce *et al.*, 1988), PDGFR (Matsui *et al.*, 1989), flk2/flt3 receptor (Dosil *et al.*, 1993) and the c-Kit receptor (Hu *et al.*, 1995; Kitayama *et al.*, 1995). The ability of RTK to function in these cells suggests that some of the substrates involved in the transduction of IL-3 and/or GM-CSF induced signalling may be the same as those recruited by the RTK. In relation to the c-Kit receptor it has been shown that although SLF and IL-3 phosphorylate unique substrates of their own, both receptors are

able to induce phosphorylation of common cytoplasmic molecules such as MAPK, Raf-1 kinase, and some other unidentified molecules (Hallek *et al.*, 1992; Okuda *et al.*, 1992; Welham & Schrader, 1992) which may be involved in cellular proliferation. RTK and their ligands could not support proliferation of the IL-2 dependent T cell line, CTLL (von Rüden *et al.*, 1990, 1991); these results demonstrate that different cell types display distinct signal transduction pathways.

The diversity of responses generated from the c-Kit receptor such as cell survival, proliferation, differentiation, adhesion and chemotaxis may be dependent on different signal pathways as has been suggested by the discussion above. The response induced by the c-Kit receptor upon SLF stimulation may be influenced by: the set of signalling molecules present or absent within a particular cellular background, the degree of stimulation by varying SLF concentrations or the isoform of SLF (see section 1.5.3), the level of the receptor expressed at the cell surface (see section 1.8.2), the duration of activation of certain signal molecules (see section 1.8.2) or the combination of other receptors present in the cell which may induce heterodimerization (see section 1.8.1).

1.7.4: Downmodulation of c-Kit expression/activation

Following ligand binding it has been shown that the receptor-ligand complexes are endocytosed via clatherin coated pits. The receptor is then either recycled to the cell surface, targeted to lysosomes where it is degraded, or degraded by polyubiquitination. Internalisation of the receptor-ligand complex can be seen as a way of reducing the life span of the receptor and thereby downmodulating ligand-induced activation of the receptor. Downmodulation of the c-Kit receptor induced by SLF stimulation has been reported by several groups and has been found to be the result of number of different, but not mutually exclusive, mechanisms.

The binding of soluble SLF to the c-Kit receptor has been shown to induce c-Kit/SLF complex internalisation in mast cells and the megakaryocytic cell line, M07e (Yee *et al.*, 1993, 1994a; Miyazawa *et al.*, 1994; Adachi *et al.*, 1995) resulting in reduced levels of the c-Kit receptor on the cell surface. Internalisation could also be induced by phorbol 12-myritate 13-acetate (PMA), a PKC activator, with a concomitant decrease in c-*kit* RNA

expression seen (Adachi *et al.*, 1995; Ogawa *et al.*, 1995). Asano *et al.*, (1993) demonstrated that decreased c-*kit* mRNA expression in a human erythroleukaemia cell line, HEL, upon 12-0-tetradecanoylphorbal-13-acetate (TPA) stimulation was a result of posttranscriptional mechanisms involving induction of a RNA destabilising protein. It is interesting to note that, since SLF exists as both a soluble and membrane-bound ligand, the binding of c-Kit to membrane-bound SLF may prevent c-Kit/SLF internalisation. Recently this was shown to be the case; membrane-bound SLF was able to prolong the life span of the c-Kit receptor and induce more persistent tyrosine kinase activation in comparison to soluble SLF (Miyazawa *et al.*, 1995), which may explain the biological differences seen between these two SLF isoforms (discussed in section 1.5.3).

Internalisation and degradation of the c-Kit/SLF complex has been shown to be reduced in kinase defective c-Kit receptors and receptor ubiquitination was shown to be dependent on kinase activity (Yee *et al.*, 1994a; Miyazawa *et al.*, 1994). Similar results have been obtained with the CSF-1 and PDGF receptors (Carlberg *et al.*, 1991; Mori *et al.*, 1992, 1993). The c-Kit receptor can also be downmodulated by the shedding of the extracellular domain (100 kd) from the cell surface. This latter mechanism is induced by PKC dependent and independent pathways (Yee *et al.*, 1993; Brizzi *et al.*, 1994; Adachi *et al.*, 1995) (see also section 1.7.5) and it is independent of kinase activity which is in agreement with results obtained with the CSF-1R (Yee *et al.*, 1994a; Downing *et al.*, 1989b).

PKC has also been shown to downregulate the SLF-induced activation of the c-Kit receptor by inhibiting tyrosine autophosphorylation and the phosphorylation of the associated PI 3'-K subunit p85. This is achieved by serine phosphorylation of the receptor as previously described in section 1.7.3. (Blume-Jensen *et al.*, 1993, 1994).

Downregulation of c-Kit can also be induced by several cytokines such as IL-3 and GM-CSF in murine bone marrow derived mast cells (Welham & Schrader, 1991), IL-4 in the human mast cell line, HMC-1, and AML cells (Sillaber *et al.*, 1991), transforming growth factor-β (TGF-β) in CD34+ cells (Sansilvestri *et al.*, 1995) and erythroid differentiation factor/activin A in murine erythroleukaemia cells (Hino *et al.*, 1995)

1.7.5: Soluble c-Kit Receptor

Soluble c-Kit receptors containing the entire extracellular domain have been found 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). Soluble c-Kit receptors are presumably produced as a result of proteolytic cleavage of either the full length (Turner *et al.*, 1995, Yee *et al.*, 1993, 1994) or from a truncated transmembrane c-Kit receptor (lacking the intracellular domain) (Brizzi *et al.*, 1994) resulting in ~100 kd protein. The site of cleavage or the proteases involved have not yet been identified but may be similar to those used to generate soluble SLF (discussed in section 1.5.1). Soluble forms of many of the RTKs and cytokine receptors exist produced by proteolytic cleavage or from alternative transcripts (Ullrich *et al.*, 1984; Downing *et al.*, 1989b; Raines *et al.*, 1991; Tiesman *et al.*, 1993; Ulich *et al.*, 1993; Carter *et al.*, 1994; Lin *et al.*, 1995). Native and recombinant soluble c-Kit receptors are able to bind SLF, acting as antagonists *in vitro* (Flanagan *et al.*, 1990; Lev *et al.*, 1992c; Liu *et al.*, 1993; Kendall *et al.*, 1993; Turner *et al.*, 1995). In light of this it is possible that in serum soluble c-Kit receptors (320 ng/ml) (Wypych *et al* 1995) are able to bind to soluble SLF (3 ng/ml) (Langley *et al.*, 1993) thereby regulating the c-Kit/SLF interactions. Soluble c-Kit could also bind to membrane-bound SLF either blocking the binding of functional c-Kit receptors or triggering a response within the cell expressing membrane-bound SLF (if the cytoplasmic tail of SLF is involved in signalling (see section 1.5.2). Cleavage of the c-Kit receptor could also leave the extracellular domain bound to the membrane-associated SLF, enabling the mast cell to migrate away (Yee *et al.*, 1993). This event is similar to that described in section 1.5.3 however this involved the cleavage of membrane-associated SLF bound to c-Kit receptors. It may also be possible for soluble c-Kit to produce non-functional heterodimers with wild type c-Kit receptors on the cell surface, resulting in a dominant-negative effect similar to that seen in heterozygous *W* mutant mice (described in section 1.4), although crosslinking experiments in the presence of SLF suggest that this does not occur (Turner *et al.*, 1995).

1.8 Mechanisms inducing signalling diversity by the RTK

1.8.1: Heterodimerization

Heterodimerization of mutant receptor or ligand subunits have provided us with evidence on how RTK function to transmit their signal. The heterodimerization of kinase-defective receptor subunits with wild type receptor molecules have displayed dominant-negative effects similar to the underlying mechanism of mutant c-Kit receptors in the heterozygous *W* mutant mice (Chabot *et al.*, 1988; Reith *et al.*, 1990; Tan *et al.*, 1990)(see section 1.4). For example the heterodimerization of EGFR or PDGFR with cytoplasmic deleted mutant receptors have been shown to suppress signalling of the wild type receptor, acting as an antagonist by preventing transphosphorylation (Kashles *et al.*, 1991; Ueno *et al.*, 1991; Spivak-Kroizman *et al.*, 1992). They have shown the importance of dimerization as a prerequisite to the induction of transphosphorylation and subsequent association and activation of signalling molecules. The PDGF ligand, like others binding to type III RTK, exists as a dimer. Heterodimerization of mutant subunits unable to bind to the receptor with functional subunits supports the data already described in section 1.7.2 (Blume-Jensen *et al.*, 1991; Heldin *et al.*, 1989) that the bivalency of the ligand is important for stable dimerization of the receptor and to produce high affinity ligand binding (Vassbotn *et al.*, 1993).

Heterodimerization can also increase the repertoire of signalling pathways a receptor complex can activate. Heterodimerization between two different ligand isoform monomer subunits (for example PDGF-A and B) or two functional receptors belonging to the same family (for example members of the ErbB family) can occur, resulting in an increase in signal diversity. PDGF occurs as three different isoforms, which are made up as disulphide bonded homo- and heterodimers, composed of homologous A and B polypeptide chains, PDGF-AA, PDGF-AB and PDGF-BB. The PDGF isoforms exert their biological effects by binding with different affinities to two distinct cell surface receptors; the αPDGF receptor binds all three isoforms with high affinity whereas the βPDGF receptor only binds PDGF-BB with high affinity and PDGF-AB with lower affinity. The α and βPDGFR are able to activate unique and common signalling pathways. The subsequent biological outcome depends on the

combination and level of expression of the different PDGF isoforms and/or PDGFR expressed within the cell. This can explain how one receptor is able to provide a diverse range of functions within different cell types (for review see Claesson-Welsh, 1994; Lemmon and Schlessinger, 1995, Heldin, 1995 and references therein).

The increase in the recruitment of signalling molecules that can associate with receptor complexes has also been shown through the ErbB family (RTK I), in which different receptors belonging to this group are able to heterodimerize. In addition their ligands display the ability to cross-bind with other receptors belonging to this family, overall providing a model of trans-regulation. The ErbB family consists of ErbB1 (EGFR), ErbB2 (HER-2/*neu*), ErbB3 (HER-3) and ErbB4 (HER-4) to date (Lemmon and Schlessinger, 1994). The EGFR is able to bind several ligands which include EGF, TGF-α, amphiregulin, heparin-binding EGF and betacellulin. The ligand to ErbB2 at present is not known, (although many claims of its isolation have been made) and the ligand for ErbB3 and 4 is *neu* differentiation factor (NDF) or the human equivalent, heregulin, which has 10 isoforms produced by alternative splicing that bind with different affinities to their receptors (Wen *et al.*, 1994). It has been shown that the ErbB1 is able to heterodimerize with the ErbB2 receptor and that this is induced by EGF binding to ErbB1 as EGF does not bind to ErbB2 receptor. This heterodimerization induces phosphorylation of the ErbB2 receptor by transphosphorylation (Goldman *et al.*, 1990; Wada *et al.*, 1990). Heterodimerization has also been shown to occur between ErbB1 or ErbB2 with ErbB3 or ErbB4 due to the binding of NDF to the latter two receptors, since it does not bind to ErbB1 or ErbB2 (reviewed in Lemmon and Schlessinger, 1994; Heldin 1995). The binding affinity of EGF to a NDF occupied heterodimer is reduced therefore heterodimerization can favour the binding of one ligand over another and this may avoid excessive or even opposing growth regulatory signals. Overexpression of one receptor over the other will force heterodimerization to occur resulting in one of the scenarios discussed in this section (Karunagaran *et al.*, 1995).

If both receptor molecules within the heterodimer are able to associate with different SH2 signalling molecules then this will also increase the diversity of the response. This is indeed the case with the α and βPDGFR which not only associate and activate both common

and unique molecules as homodimers but upon heterodimerization autophosphorylate different tyrosine residues within the receptor complex enabling the association with a another set of SH2 molecules (Lemmon and Schlessinger, 1994; Heldin, 1995). Similarly the EGFR is able to associate with other SH2 containing molecules when complexed with other members of the RTK I family. The ErbB3 receptor presents an interesting set of events in this regard as it has an impaired tyrosine kinase activity but nevertheless possesses several putative consensus sequences for the binding of the p85 subunit of PI 3'-K unlike the other three members of this family. Heterodimerization of ErbB3 with other members of the RTK I receptor would then enable coupling to PI 3' K (Carraway *et al.*,1995).

As discussed in section 1.3 several isoforms of the c-Kit receptor also exist, however it has not yet been shown whether these isoforms are able to heterodimerize. It is possible that heterodimerization may be able to alter the biochemical events achieved upon c-Kit activation or trans-regulate SLF-dependent and/ or -independent responses in a similar fashion to those achieved by other RTK described in this section.

1.8.2: Receptor levels

Receptor densities have been shown to determine the biological response of a cell by influencing the signalling events that follow ligand-stimulation. EGF stimulation of several carcinoma and transfectant cell lines expressing high levels of the EGFR results in the inhibition of proliferation, in contrast to cells expressing lower receptor levels (Kawamoto *et al.*, 1984; Riedel *et al.*, 1987; Lupu *et al.*, 1990). Inhibition of proliferation was associated with an increase in tyrosine kinase activity of the EGFR (Kawamoto *et al.*, 1984). This phenomenon may be explained by the recent data demonstrating that overexpression of the EGF or insulin receptors in the phaeochromocytoma PC12 cells results in sustained activation of MAPK which is associated with the differentiation of the cells. Low receptor levels on the other hand result in transient activation of MAPK leading to a proliferative cellular response (Dikic *et al.*, 1994; Traverse *et al.*, 1994; Marshall, 1995). These studies also revealed that EGF stimulation of cells overexpressing EGFR inhibited their cell division resulting in an

inhibition of proliferation analogous to the results of Kawamoto *et al.*, (1984) described above.

The amplitude or duration of the signal generated as a consequence of receptor density may also result in the activation/association of other signalling molecules that interact with the receptor with low affinity. For example, as previously mentioned in section 1.7.3, the association of the EGFR with PLC-γ1 required the presence of high receptor levels (Wahl *et al.*, 1988; Margolis *et al.*, 1989). This may result in a different cellular response either due to the induction of new signalling pathways or the alteration of normal ones. The strength of the signal generated may also be the underlying mechanism involved in the induction of transformation by many RTK when expressed at high levels, as will be discussed in section 1.9.3.3 below. It is possible that different cellular responses generated from the same receptor can also be achieved by variation in the concentration of ligand available for binding (Kawamoto *et al.*, 1984). Thus, variations in receptor and/or ligand concentrations may alter the duration or strength of the signal and/or induce the interaction with different signalling molecules. It has been demonstrated that higher concentrations of SLF are required for the proliferation of mast cells than for maintaining their survival (Yee *et al.*, 1994b) and that lower levels are required for cell adhesion than for proliferation (Kinashi & Springer, 1994).

The level of surface expression of the c-Kit receptor is regulated during haemopoiesis. It is possible that similar mechanisms as those described in this section may determine the biological response (for example cell survival, proliferation, chemotaxis, adhesion or differentiation) upon SLF stimulation. *W* mutations resulting in reduced levels of functional c-Kit (eg. *W/+*, W^{44}, W^{57} and W^{sh}) have already implied that c-Kit receptor densities may differentially affect certain attributes of these mutant mice (see section 1.4).

1.9 Transformation induced by RTKs

1.9.1: The assays used to demonstrate transformation

The activation of genes captured by retroviruses was originally brought to our attention by their ability to produce tumours in animal hosts (as discussed in section 1.1). Subsequently *in vitro* assays were used to demonstrate this phenomenon in cultured cells.

Introduction of oncogenes, either by infection or transfection, into cultured fibroblast monolayers (eg of NIH/3T3 cells) results in characteristic phenotypic changes indicative of transformation. These cells normally display flattened morphology and grow in an anchorage-dependent and contact-inhibited manner. Introduction of an oncogene may result in all or some of the following phenotypic alterations: morphological changes resulting in a refractile, spindly shaped appearance and irregular growth of the cells compared to the opaque, flat regular growth of untransformed cells; the ability to produce foci due to the loss of contact inhibition; the ability to grow in semi-solid agar due to the loss of anchorage dependence; the ability to grow in low serum conditions; and the ability to induce tumours in *nude* mice (Temin *et al.*, 1958; Macpherson *et al.*, 1964; Smith *et al.*, 1971; Shin *et al.*, 1975).

The induction of focus-formation in NIH/3T3 cells was used as the initial step toward cloning the genes responsible for transformation or tumourigenesis when cells or animals, respectively, were exposed to chemical carcinogens. The transforming rat *neu* gene in nitrosoethylurea-induced rat neuroblastomas (Shih *et al.*, 1981; Padhy *et al.*, 1982) and the human *ras* gene in the bladder carcinoma cell line, EJ (Shih *et al.*, 1982), were cloned in this way. DNA was extracted from non-murine tumour cell lines, primary tumours and chemically transformed cells and introduced into NIH/3T3 cells. The monolayer was then scanned for focus-formation indicating the presence of a transforming gene within these cells. These cells could then be isolated from the other non-transformed cells and their DNA extracted for further rounds of transfection resulting in the isolation of the responsible gene from the murine background (Watson *et al.*, 1987b). Although this assay aided in the cloning of several oncogenes, it was only successful for those genes which were not restricted to a tissue or species-specific recipient cell type. Also, it did not detect oncogenes that acted as recessive or weakly transforming genes (Bishop 1983; Land *et al.*, 1983a). Furthermore, certain genes are only able to induce some of the alterations associated with transformation, and those which are unable to induce foci would not be detected by this assay, although these same genes are able to induce tumours in mice (Blair *et al.*, 1982; Fasano *et al.*, 1984). This shows the need to assay the cells for several of the transformation phenotypes mentioned above. Many of the

genes uncovered from tumours and transformation induced by chemical carcinogens were discovered to be the same genes transduced by retroviruses.

Immortalised cell lines such as NIH/3T3, Rat-1 and -2, and several embryo-derived fibroblast cell lines from a variety of species have been found to be appropriate in taking up DNA and also display the characteristic transformed phenotypes described above upon harbouring an oncogene. In contrast, primary cells when exposed to the same oncogenes fail to transform. NIH/3T3 cells and their equivalent are immortal and have already undergone a number of *in vitro* passages compared to primary cells. It is known that carcinogenesis is a multistep process and that oncogene co-operation is required to induce transformation. It is likely that NIH/3T3 cells have already undergone some of these steps prior to the introduction of the oncogene during immortalisation. This was confirmed by data demonstrating that the introduction of the human *ras* oncogene into primary embryo fibroblasts did not induce tumourigenicity, whereas it did so in immortalised cells. The co-transfection of *ras* and *myc* however was able to transform primary cells (Land *et al.*, 1983a, b).

1.9.2: Modes of Transformation by Growth Factor RTK

Captured cellular genes of retroviruses in the majority of cases have accumulated alterations which activate them into a more potent oncogene, such as the truncation of the NH_2 and COOH-termini, or point mutations within the molecule (see section 1.1). However, there are also cases in which mere overexpression of the normal cellular gene under the control of a strong retroviral promoter is sufficient to induce transformation (see sections 1.1 and 1.9.3.3.). Another scenario which can induce transformation with respect to growth factor receptors is the co-expression of the corresponding ligand within the same cell, resulting in constitutive activation of the receptor due to autocrine stimulation (see section 1.9.3.2).

As discussed in section 1.7.3, several factor-dependent cell lines possess the necessary signal molecules to allow the function of ectopically expressed growth factor receptors. These cells also provide a more suitable background than NIH/3T3 cells and their equivalents to address whether growth factor or receptor-related oncogenes/proto-oncogenes are able to

induce transformation by either causing factor-independence, differentiation, or tumourigenicty in mice.

1.9.3: Transforming potential of the c-*kit* gene

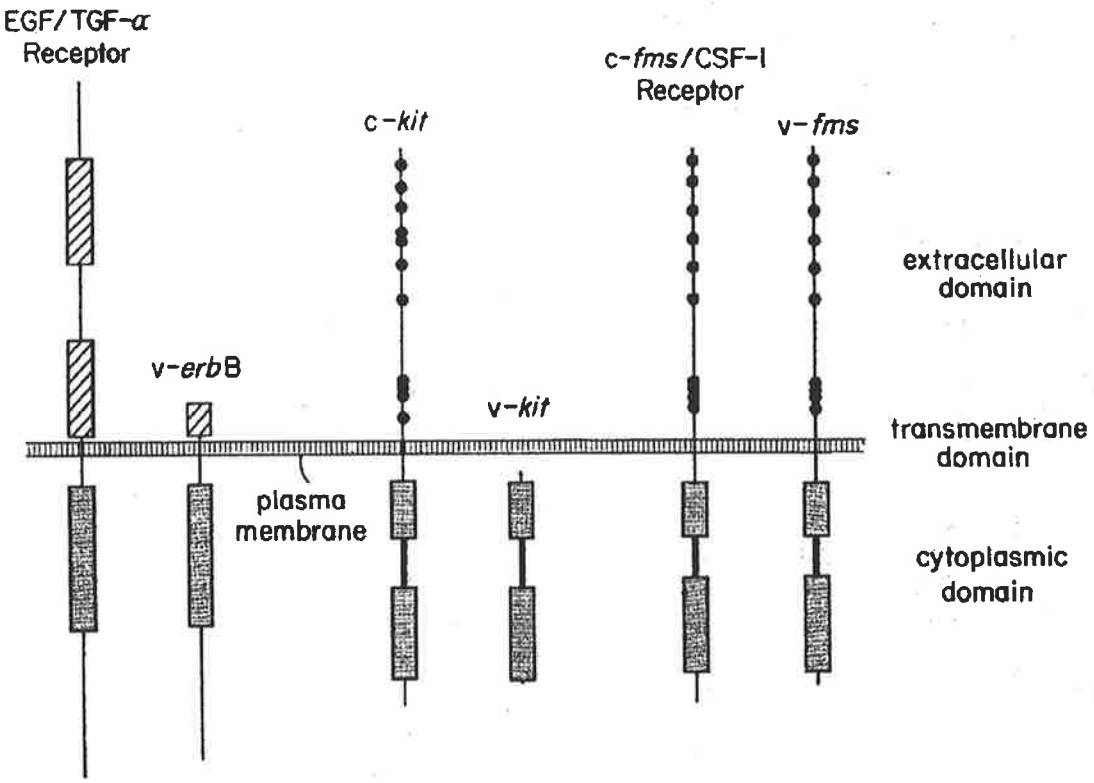
1.9.3.1: Genetic alterations that activate the c-Kit receptor

Transduction of c-*kit* by the HZ4-FeSV to produce the v-*kit* oncogene enabled it to produce tumours in cats and induce a transformed phenotype in NIH/3T3 cells (Besmer *et al.*, 1986b). The most dramatic differences between v-*kit* and c-*kit* were the removal of the sequences encoding the entire extracellular and transmembrane domains (v-*kit* is attached to the membrane via gag-linked myristoylation) and 49-50 amino acids from the carboxyl tail (see Figure 1.9.3.1) (Yarden *et al.*, 1987a; Qui *et al.*, 1988; Herbst *et al.*, 1995a). Removal of part of the extracellular domain and carboxyl tail have also occurred during the transduction of the v-*erbB* gene by the AEV (Downward *et al.*, 1984; Ullrich *et al.*, 1984) and removal of part of the carboxyl tail was also seen in the v-*fms* gene harboured by the SM and HZ5-FeSV (McDonough *et al.*, 1971; Besmer *et al.*, 1986a; Coussens *et al.*, 1986) (see Figure 1.9.3.1). These alterations unleash the oncogenic potential of the receptors (Gazit *et al.*, 1986; Wheeler *et al.*, 1986, 1987; Dibb *et al.*, 1990; Shounan *et al.*, 1995).

Alterations of the extracellular domain of the v-*erbB* and v-*kit* gene products results in the loss of the ability to bind ligand. As a consequence these receptors are constitutively activated resulting in transformation which is factor-independent (Gazit *et al.*, 1986; Shounan *et al.*, 1995; Majumder *et al.*, 1990). The v-*fms* receptor still possesses all of its extracellular domain and is therefore able to bind ligand but contains three point mutations within this domain compared to its normal counterpart c-*fms* (CSF-1R). The mutations at residues 301 and 374 when introduction into the c-*fms* receptor contribute to its transforming ability of NIH/3T3 and Rat-2 cells in the absence of ligand (Roussel *et al.*, 1988; Woolford *et al.*, 1988) They also contribute to inducing factor-independent growth and tumourigenicity of the IL-3 dependent cell line, 32D (Pierce *et al.*, 1990). Similarly a point mutation in the transmembrane domain of the HER-2/*neu* receptor renders it constitutively active resulting in its ability to induce transformation (Bargmann *et al.*, 1988; Weiner *et al.*, 1989).

Figure 1.9.3.1. Diagram taken from Yarden *et al.*, (1987a) and depicts the unaltered structures ot the RTK EGF, CSF-1 and c-Kit. Diagram also shows the altered structures produced as a result of part of the respective cellular *erbB*, *fms* and *kit* sequences being captured by the retroviruses listed in Table 1.1.

The hatched regions depict cysteine-rich regions and the closed circles represent individual cysteine residues in the type III receptors. The single kinase domain of the type I receptors and the split kinase domain of the type III receptors are represented by the shaded regions.


EGF/TGF-α
Receptor
c-kit
c-fms/CSF-1
Receptor
v-fms
extracellular
domain
v-erbB
v-kit
transmembrane
domain
plasma
membrane
cytoplasmic
domain

The removal of part of the carboxyl tail of all these receptors is analogous to that which occurred in the v-*src*/c-*src* pair discussed in section 1.1 in which the deletion resulted in the removal of Y_{527} which served as a negative regulator of kinase activity. Removal of this residue resulted in the oncogenicity of c-*src* (see section 1.1). Similarly the carboxyl deletion in the v-*erbB* and v-*fms* oncogenes results in the removal of equivalent tyrosine residues. Substitution/removal of these residues or domain swapping of the viral COOH-terminal tail onto the normal receptors results in increased kinase activity. In the case of the c-*fms* receptor, mutation of the Y_{969} in combination with the point mutations in the extracellular domain (301 and 374) are required to achieve a fully transformed phenotype (Roussel *et al.*, 1987, 1988; Pierce *et al.*, 1990; Downward *et al.*, 1984). In the v-*kit* oncogene the carboxyl deletion results in the removal of Y_{936} (human; 934 in mouse), however in contrast to c-*fms* and c-*erbB,* substitution of this residue in the c-Kit receptor with phenylalanine resulted in a decrease in transformation potential (Herbst *et al.*, 1995a).

Apart from the NH_2 and COOH-terminal truncations of v-*kit,* comparison of the human and murine c-*kit* and feline v-*kit* sequences revealed that amino acid substitutions and deletions occurred in v-*kit* (Yarden *et al.*, 1987a, figure 3; Qui *et al.*, 1988, figure 2). Whether these differences were mutational or due to species divergence had not been determined until recently when feline v-*kit* and c-*kit* sequence comparisons were made (Herbst *et al.*, 1995a). These workers showed that Y_{569} and V_{570} in the juxtamembrane domain were missing in v-*kit* but were present in each of the feline, human and murine c-*kit* sequences. v-*kit* also had a G instead of a D at position 761, in the kinase insert domain, compared to c-*kit* (Herbst *et al.*, 1995a, figure 2). All other differences between human or murine c-*kit* and v-*kit* could be attributed to species divergence. Analysis of the consequences of these mutations was carried out by introducing the mutations into a chimeric receptor involving the fusion of the extracellular domain of the human EGFR to the transmembrane and intracellular domains of the human c-Kit receptor. Constructs containing these mutations individually or in combination were expressed in the human kidney embryo cell line, 293T (Herbst *et al.*, 1991, 1995a). In the absence of EGF there was no autophosphorylation of any of the mutants demonstrating that the constitutive phosphorylation seen in v-*kit* is probably a result of its loss

of the extracellular domain. Individually the mutants did not alter the autophosphorylation of the receptor. However the deletion of both Y_{569} and V_{570} and the exchange of D_{761} to G significantly impaired autophosphorylation and when the last 49 amino acids were also removed from this mutant receptor the effect was more pronounced. Nevertheless, the deletion of Y_{569} and V_{570} resulted in a 3 fold higher ability to induce anchorage independent growth of NIH/3T3 cells in the presence of EGF compared to the control EGF/Kit receptor. Y_{568} corresponds to Y_{579} and Y_{559} of the human PDGFR and CSF-1R respectively. These Y residues in the latter two receptors have been shown to be involved in the binding of tyrosine kinase substrates belonging to the c-*src* family (Courtneidge *et al.*, 1993). The deletion of Y_{568} in the c-Kit receptor may therefore affect its ability to bind to similar c-*src* substrates, possibly contributing to its oncogenic activation. So, in addition to the extracellular domain deletion, the Y_{569} and V_{570} deletion may increase the transforming ability of v-*kit* (Herbst *et al.*, 1995a).

As well as the mutations found in the v-*kit* gene product, two point mutations which induce constitutive activation of the c-Kit receptor, resulting in factor-independence, have been described in several mast cell lines. These mutations involve the substitution of a glycine (Gly) for a valine (Val) at position 560 and the substitution of a Val for an asparagine (Asp) at position 816 in the human mast cell line, HMC-1 (Furitsu *et al.*, 1993). A point mutation equivalent to the latter mutation was also found in the murine and rat mastocytoma cell lines, P815 and RBL-2H3, respectively (Tsujimura *et al.*, 1994, 1995). Gly_{560} is located in the juxtamembrane region of the intracellular domain and $Asp_{816/814/817}$ is located in the phosphotransferase domain of the human/murine/rat c-Kit receptors, respectively. Both these mutations independently induced phosphorylation of the c-Kit receptor expressed in 293T cells and the IL-3 dependent cell lines, FDC-P1 and Ba/F3, in the absence of SLF (Furitsu *et al.*, 1993; Kitayama *et al.*, 1995). The c-Kit receptor possessing G_{560} was able to undergo dimerization in the absence of ligand however c-Kit Asp_{816} was not. The former situation is similar to the point mutation in the transmembrane domain of the *HER-2/neu* oncogene product, which causes dimerization and activation of its intrinsic tyrosine kinase in the absence of ligand (Bargmann *et al.*, 1988; Weiner *et al.*, 1989). Expression of these mutants in

FDC-P1 and Ba/F3 cells rendered them factor-independent as well as tumourigenic in *nude* mice (Kitayama *et al.*, 1995). It has recently been shown that the Asp_{816} substitution is also present in some patients with systemic mastocytosis (Nagata *et al.*, 1995). Interestingly, isolation of a constitutively activated mutant of the CSF-1R, generated by random mutagenesis, was recently discovered to be a result of an equivalent Asp substitution as found in the c-Kit receptor (Glover *et al.*, 1995).

1.9.3.2: Autocrine transformation

Many tumour cells have been shown to be able to secrete growth factors to which they can respond since they also possess the corresponding receptors. This autocrine secretion of growth factors can explain how these tumour cells require less growth factor supplements and acquire a growth advantage over normal cells (reviewed in Sporn *et al.*, 1985).

The v-*sis* oncogene provided a model demonstrating that autocrine stimulation could be oncogenic. v-*sis* is the transforming gene of the simian sarcoma virus (SSV) isolated from a fibrosarcoma of a woolly monkey (see Table 1.1). The cellular homologue of v-*sis* encodes the βPDGF chain (c-*sis*) (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983). v-*sis* or c-*sis* gene products can only transform cells which also express the PDGFR hence creating an autocrine loop of constitutive stimulation of PDGFR (Gazit *et al.*, 1984; Huang *et al.*, 1984). The interaction between v-*sis* gene products and PDGFR has been shown to occur at the cell surface upon secretion of the v-*sis* product, and α-PDGF antibodies have been shown to inhibit cell growth and reverse transformation (Huang *et al.*, 1984; Johnsson *et al.*, 1985). However it has also been demonstrated that in certain SSV-transformed cells antibodies cannot inhibit, and this correlates with undetectable levels of v-*sis* protein being secreted into the medium (Huang *et al.*, 1984; Robbins *et al.*, 1985). It was demonstrated that *sis* gene products and the PDGFR can interact intracellularly and activate a mitogenic response prior to the molecules reaching the surface (Huang *et al.*, 1988; Keating *et al.*, 1988; Bejcek *et al.*, 1989). The co-expression of PDGF and PDGFR has also been shown in human lung carcinomas and malignant glioma tissue (Antoniades *et al.*, 1992; Hermansson *et al.*, 1992). Other examples in which autocrine stimulation induces transformation in cultured fibroblasts

and in human tumours are TGF-α stimulating co-expressed EGFR (Sporn *et al.*, 1985; Rosenthal *et al.*, 1986; Derynck *et al.*, 1987; Fan *et al.*, 1994) and the stimulation of the CSF-1R due to secretory and/or intracellular autocrine stimulation by CSF (Rettenmier *et al.*, 1987; Rohrschneider *et al.*, 1989). Co-expression of this latter receptor/ligand pair has been detected in human myeloid leukaemic cells (Rambaldi *et al.*, 1988).

The c-Kit receptor has also been implicated in transformation and carcinogenesis due to co-expression with SLF. It has been suggested that NIH/3T3 cells ectopically expressing c-Kit are able to grow in an anchorage independent manner due to stimulation by endogenously produced SLF (Alexander *et al.*, 1991). Co-expression of c-Kit and SLF has been found in small-cell lung cancer (Hibi *et al.*, 1991; Turner *et al.*, 1992), breast and gastric carcinomas, melanoma cell lines, (Turner *et al.*, 1992) some cases of uterine cancer (Inoue *et al.*, 1994) and malignant testicular tissue (Strohmeyer *et al.*, 1995), human malignant glioma cell lines (Stanulla *et al.*, 1995) and in myeloid leukaemia (Caceres-Cortes *et al.*, 1992; Pietsch 1993). In an *in vivo* transgenic mouse model in which a Leydig cell tumour develops, co-expression of c-Kit and SLF has also been demonstrated (Kondoh *et al.*, 1995). Broudy *et al.* (1994) demonstrated that co-expression could also occur in normal human endothelial cells and that stimulation with exogenously added SLF did not increase the proliferative response of these cells and the addition of an antagonistic α-c-Kit mAb did not inhibit proliferation. Similar results were described with melanoma and gastric carcinoma cell lines (Turner *et al.*, 1992). These results suggest that SLF and c-Kit may also be involved in an intracellular autocrine mechanism. It has also been shown for the c-Kit expressing, factor-dependent cell lines, UT-7 and M07e, that the interaction with exogenously supplied SLF was able to induce the production of GM-CSF for which the cells also possess receptors, thereby stimulating an autocrine loop for another receptor/growth factor pair (Kiss *et al.*, 1993; Sasaki *et al.*, 1995).

1.9.3.3: Overexpression of RTK

It has been shown for several proto-oncogenes that mutational activation of the gene is not necessary for the acquisition of oncogenicity (see section 1.1). Overexpression of

unaltered RTKs, such as EGFR or the closely related HER-2/*neu*, due to the genes encoding these receptors being under the control of a retroviral LTR or as a result of gene amplification, have also been shown to induce transformation of NIH/3T3 cells and to induce tumours in *nude* mice. In contrast, low levels of expression either as a result of transcriptional activation under a less efficient promoter (SV40) or low expressing clones, were unable to induce transformation (Di Fiore *et al.*, 1987a; Hudziak *et al.*, 1987; Velu *et al.*, 1987). In the case of the EGFR transformation was dependent on factor, either exogenous EGF (Di Fiore *et al.*, 1987a; Velu *et al.*, 1987) or by autocrine stimulation with co-expressed transforming growth factor (TGF)-α which also acts through the EGFR (Di Marco *et al.*, 1989). It is interesting to note that expression of TGF-α in NIH/3T3 cells expressing low endogenous levels of EGFR were insufficient to induce transformation (Finizi *et al.*, 1987) and therefore the combination of autocrine stimulation and receptor overexpression were required. Overexpression of a chimeric EGF/Kit receptor and a full length murine c-Kit receptor also induced factor-dependent transformation of NIH/3T3 cells (Lev *et al.*, 1990; Alexander *et al.*, 1991). In the case of the HER-2/*neu* receptor, exogenously added ligand was not required to induce transformation, however it is possible that the NIH/3T3 cells produce a ligand for HER-2/*neu* (Lee *et al.*, 1989) and therefore high receptor levels and autocrine stimulation may indeed be necessary. However expression of another member of the ErbB family in conjunction with its ligand may be present, enabling heterodimerization with the overexpressed HER-2/*neu* receptors (see section 1.8.1). Alternatively, Brandt-Rauf *et al.*, (1990) described a model suggesting that ligand-independent dimerization could occur at high receptor levels and it was recently shown that purified HER-2/*neu* produced in insect cells could self-dimerize when at high concentrations in the absence of ligand (Samanta *et al.*, 1994). Similar results have been demonstrated for the PDGFR (Herren *et al.*, 1993). Therefore overexpression of the normal receptor maybe sufficient to induce factor independent dimerization analogous to the mutant HER-2/*neu* receptor possessing a point mutation in the transmembrane domain (Weiner *et al.*, 1989). Overexpression of RTKs leads to an increase in receptor oligomerization and enhanced kinase activity (as explained in section 1.8.2), which correlates with the induction of transformation.

Overexpression of the normal c-Kit receptor may also induce tumourigenicity of factor-dependent cells in *nude* mice but at a low frequency (Kitayama *et al.*, 1995) and may induce factor independence in factor dependent cell lines (Hu *et al.*, 1995). Similarly, it has been shown that overexpression of the unaltered CSF-1R in FDC-P1 cells can lead to factor independence (Kato *et al.*, 1989).

Amplification and/or overexpression of either the EGFR or HER-2/*neu* receptors have been reported in a number of human malignancies including adenocarcinoma, colon, breast, ovarian, (Slamon *et al.*, 1987, 1989; Paik *et al.*, 1990; Liu *et al.*, 1992), salivary gland (Press *et al.*, 1994), and non-small cell lung carcinomas (Kern *et al.*, 1990). In particular, overexpression of the HER-2/*neu* proto-oncogene has been shown to correlate with poor prognosis and resistance to chemotherapy in patients with breast and/or ovarian cancers (Slamon *et al.*, 1987, 1989) and this discovery has resulted in the development of a number of new therapeutic regimens for certain groups of patients (Hudziak *et al.*, 1989, reviewed in Shepard *et al.*, 1991; Carter *et al.*, 1992; Shalaby *et al.*, 1992; Beckmann *et al.*, 1993; Katsumata *et al.*, 1995 and references therein).

1.9.3.4:c-Kit expression in leukaemogenesis

Ashman *et al.*, (1988) reported that 25% of a group of AML patients studied expressed high levels of c-Kit on their blast cells assessed by the binding of an α-c-Kit mAb, YB5.B8 (Gadd *et al.*, 1985; Lerner *et al.*, 1991) and that these patients had a poor prognosis. These results were obtained prior to the demonstration that YB5.B8 detects c-Kit, and are analogous to results obtained with the HER-2/*neu* proto-oncogene in patients with breast/ovarian cancer (section 1.9.3.3). Recent studies using more sensitive detection systems indicate that c-Kit is not appreciably overexpressed in AML and is frequently downregulated relative to normal haemopoietic progenitor cells (Cole *et al.*, 1996). Similarly downregulation rather than overexpression of c-Kit has been reported in melanoma (Natali *et al.*, 1992). c-Kit expression was reported not to be a prognostic indicator in childhood AML (Smith *et al.*, 1994) or in another study of adult AML (Reuss-Borst *et al.*, 1994).

Recently, it has been shown that c-Kit can play a role in the progression of leukaemia in a model system. Constitutive expression of the murine c-Kit receptor in an IL-3-dependent cell line, 32D, as would be analogous to the persistent expression seen on AML blast cells as a result of differentiation arrest, was shown to induce leukaemia in mice. High levels of expression of c-Kit were not essential, however the level of c-Kit expression correlated with the aggressiveness of the disease (Hu *et al.*, 1995). Growth of the cells is presumably due to the interactions with membrane-bound and soluble SLF in the mice and may provide a selective growth advantage for those cells ectopically expressing the c-Kit receptor over normal progenitor cells that still have regulatory control of c-Kit (ie those that have the ability to lose c-Kit as they differentiate).

1.10: AIM

It had previously been shown that transformation could be induced by several RTK when the unaltered receptor was expressed at abnormally high levels. High levels of expression of these receptors are frequently associated with malignancies. Since c-Kit was also a member of the RTK family and its high expression levels had been demonstrated to correlate with the poor prognosis of a group of AML patients (Ashman *et al.*, 1988), the major aim of this project was to investigate the transforming potential of unaltered c-Kit when expressed at various levels and to evaluate the ligand-dependency of this process. Several naturally occurring c-Kit isoforms were analysed to determine whether they differed in their transforming abilities. The c-Kit isoforms were also expressed in factor-dependent lines in order to investigate their responsiveness to SLF and to analyse whether mAb against c-Kit could differentially affect SLF-driven proliferation mediated by these isoforms. Data demonstrating that membrane-bound, in contrast to soluble SLF could support the growth of FDC-P1 cells also lead to the further investigation of the differential responsiveness of these cells to the SLF isoforms.

CHAPTER 2

MATERIALS AND METHODS

2.1 TISSUE CULTURE

2.1.1 Media

To make Dulbecco's Modified Eagle's Medium (DMEM), one sachet of DMEM powder (Gibco, USA, Cat. No. 12800-017) along with 3.7 g $NaHCO_3$ (BDH, USA, Cat. No. 10247) were dissolved in 900 ml tissue culture grade Milli-Q purified water (see below). Sterile stock solutions of N-2-Hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES) (Boehringer-Mannheim, Australia, Cat. No. 737151) pH 7.2, penicillin (Sigma, USA, Cat. No. P.3032) and streptomycin sulphate (Sigma, USA, Cat. No. S 9137) were added to give final concentrations of 15 mM, 100 IU/ml and 100 μg/ml respectively. The pH was adjusted to 7 using 4 ml of 1 M HCl and made up to 1 litre with tissue culture grade Milli-Q-purified water, which had been deionised using a Milli-Q RO60 system (Millipore Corp., USA) and then further purified by passing through two beds of ion exchange resins, a carbon filter and an organic filter using a Milli-Q system (Millipore, USA). This medium was filter-sterilised using a sterivex GS 0.22 μm filter unit with filling bell (Millipore, USA, Cat. No. SVGB1010). The pH after filter-sterilisation was 7.0 - 7.4. Prior to use medium was supplemented with glutamine (BDH, Cat. No. 37107) to a final concentration of 2 mM, and 10% v/v foetal bovine serum (FBS) (Gibco, USA, Cat. No. 200-6140PJ, Batch No. 660) the latter of which had been previously heat inactivated by incubating at 56°C for 30 minutes. After 7 days of storage medium was resupplemented with glutamine (2 mM final).

RPMI 1640 medium was prepared by dissolving one sachet of RPMI 1640 powder (Gibco, Cat. No. 31800-02) along with 2 g $NaHCO_3$ in 900 ml of Milli-Q-purified H_2O. Sterile sock solutions of HEPES, streptomycin sulphate and penicillin were added to give the final concentrations as above for DMEM and the pH adjusted to 7.4 with 2.5 ml 1M HCl. The volume was made up to 1 litre and the medium filtered as above. Iscove's Modified Dulbecco's Medium (IMDM) was prepared in the same way as that described for RPMI 1640 using IMDM powder (Gibco, Cat. No. 12200-036).

Double-strength Iscove's Modified Dulbecco's Medium (ds-IMDM) was prepared by dissolving 1 sachet (1 litre) of IMDM powder (Cytosystems, Australia, Cat. No. 50-016-PA) in 390 ml tissue grade Milli-Q-purified water along with 0.2 g L-asparagine. Sterile stocks of

penicillin, streptomycin, DEAE-Dextran (Pharmacia, Sweden, Cat. No. 17-0350-01) were added to give final concentrations of 200 IU/ml, 200 μg/ml and 0.19 mg/ml, respectively. Medium was filter-sterilised as described above and kept at 4°C for up to 6 months. Prior to use heat-inactivated FBS was added to a final concentration of 20% and a 7.5% $NaHCO_3$ solution was added at 1/10 volume of ds-IMDM. The final pH of the medium was 7.1

Hank's balanced salt solution (HBSS) consisted of 0.14 M NaCl (Ajax Chemicals, Australia, Cat. No. 465), 5 mM KCl (Ajax, Chemicals, Australia, Cat. No. 383), 0.3 mM $Na_2HPO_4.12H_20$ (BDH, Australia Cat. No. 10248), 0.4 mM KH_2PO_4 (BDH, Australia, Cat. No. 10203), 4.2 mM $NaHCO_3$ (BDH, Australia, Cat. No. 10247), 5.5 mM glucose (BDH, Australia, Cat. No. 10117), 1% Phenol Red (M & B, England, Cat. No. P152/18/61) (in 0.1 M NaOH) in Milli-Q-purified H_2O. The pH of the medium was 7.4 and was sterilised by autoclaving at 130°C for 20 minutes.

Tissue Culture Phosphate Buffered Saline (TC-PBS) consisted of 0.14 M NaCl, 3 mM KCl, 8 mM $Na_2HPO_4.12H_2O$ and 1 mM KH_2PO_4 in sterile tissue culture grade Milli-Q-purified water. The pH of the solution was 7.4 and was sterilised by autoclaving.

A solution for removing adherent cells from the surface of tissue culture flasks and dishes consisted of 0.054% w/v trypsin (Difco, USA, Cat No. 0152-13-1) and 0.54 mM Ethylenediaminetetra-acetic acid (EDTA) in HBBS. The solution was filtered through a low protein binding 0.22 μm filter (Millipore, USA, Cat. No. SLGV025LS) after dissolving the trypsin powder in the EDTA/HBBS. This solution was stored at -20°C to prevent inactivation of the trypsin.

2.1.2 Cytokines

Purified recombinant murine (m) *Steel* factor (SLF) was a gift from Dr. D. Williams and colleagues (Immunex Corp., Seattle, WA). Recombinant m granulocyte macrophage - colony stimulating factor (GM-CSF) (40,000 U/ml), synthesised by yeast expressing a GM-CSF expression vector, was a gift of Drs. T. Wilson and N. Gough (Walter and Eliza Hall Institute, Melbourne, Australia). Recombinant m interleukin-3 (IL-3) ($8.3x10^5$ U/ml) synthesized by a recombinant baculovirus vector was a gift from Dr. A. Hapel (John Curtin

School of Medical Research, Canberra, Australia). Cytokine units are defined such that 50 Units results in 50% of the maximal number of colonies in soft agar cultures containing 5 x 10^4 murine bone marrow cells. Purified recombinant human (h) SLF produced in E.*coli.*, was supplied by Amgen Corporation (Thousands Oaks, CA) and mast cell growth factor (MGF) produced in yeast was provided by Immunex Corporation (Seattle, WA).

2.1.3 Cell Lines and Maintainence

The murine factor-dependent cell line, FDC-P1 (Dexter *et al.*, 1980), obtained from Dr. T. Gonda (Hanson Centre for Cancer Research, Adelaide), was cultured in DMEM containing 10% FBS with mGM-CSF (80 U/ml) or mIL-3 (300 U/ml). FDC-P1(SLF) cells were grown in either of the latter cytokines or in mSLF (50-100 U/ml). The murine factor-dependent cell line, 32D (Greenberger *et al.*, 1983), obtained from Dr. A. Hapel (John Curtin School of Medical Research, Canberra, Australia) was cultured in IMDM/5% FBS supplemented with mIL-3 (50 U/ml). The murine mastocytoma cell line, P815 (Dunn and Potter, 1957), obtained from Mr Gary Penney (Department of Microbiology and Immunology, University of Adelaide, South Australia) was cultured in DMEM/10% FBS. All cultures described above were initiated at 10^4 - 5 x 10^4 cells/ml in 25 cm^2 growth area, tissue culture flasks (Corning, USA. Cat. No. 25102-25) and passaged every 2 - 3 days.

The murine fibroblast cell lines, NIH-3T3 and ψ2 (Mann *et al.*, 1983) were obtained from Dr. T. Gonda (Hanson Centre for Cancer Research). $WCB6F_1$ +/+ 3T3-1,-2 (N1), $WCB6F_1$ *Sl/Sl* 3T3 (VSOP) and $WCB6F_1$ Sl/Sl^d 3T3-5,-6,-7 were all obtained from Dr. J. Fujita (Kyoto University, Japan) (Fujita *et al.* 1989) and the early passage (ep) NIH/3T3 cell line was obtained from Elizabeth MacMillian (Hanson Centre for Cancer Research, Adelaide, Australia) at passage 135 (p135). All fibroblast cell lines described were maintained in DMEM/10% FBS as monolayers in 75 cm^2 growth area, tissue culture flasks (Corning, USA. Cat. No. 25110-75) and were subcultured every 4 to 5 days. Cells were not allowed to become confluent to avoid spontaneous transformation, and in the case of the epNIH/3T3 line the passage number was recorded. All experiments using these latter cells were initiated with the earliest frozen stocks (p136). Fibroblast cultures were harvested by rinsing the adherent

monolayers with HBSS and then incubating at 37°C for a maximum of 2 minutes with 1 ml HBSS/1 ml Trypsin-EDTA-HBSS solution. The trypsin was inactivated by the addition of DMEM containing 10% FBS and flasks were reseeded with between 5 x 10^5-10^6 cells.

The human megakaryocytic leukaemic cell line, M07e (Avanzi *et al.*, 1988) was obtained from Dr. P. Crozier (Department of Molecular Medicine, School of Medicine, University of Auckland, New Zealand) and maintained at log phase in DMEM/10% FBS supplemented with conditioned medium produced from chinese hamster ovary cells transfected with either hGM-CSF or hIL-3 cDNA provided by Dr A. Lopez, (Department of Human Immunology, Hanson Centre for Cancer Research, Adelaide, Australia). The human erythroleukaemic cell line, HEL (Martin & Papayannopoulou, 1982), obtained from the American Type Culture Collection (ATCC) and the human mast cell leukaemic line, HMC-1 (Butterfield *et al.*, 1988), obtained from Dr. J. Butterfield (Mayo Clinic, Rochester, MN) were maintained in RPMI 1640/10% FCS

All cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cell densities were calculated using a haemocytometer and viabilities determined using trypan blue exclusion (0.8% w/v in saline). Suspension cultures were maintained for a maximum of three months and adherent cultures were maintained in culture for no longer than a month.

2.1.4 Cryopreservation of Cells

Cells were harvested at log phase and resuspended at 5 - 10 x 10^6 cells/ml in medium/10% FBS and an equal volume of cryoprotectant (30% heat-inactivated FBS, 20% analytical grade dimethyl sulphoxide (DMSO) (BDH, Merck, Vic., Australia Cat. No. 10323) and 50% RPMI) was added. 1 ml aliquots of this cell suspension were then added to 1 ml cryotubes (Nunc, Denmark, Cat. No. 3-66656) and cooled to approximately -100°C via a controlled rate freezer (Paton Industries, South Australia). The rate of freezing was 5°C/minute down to 0°C, 1°C/minute to -25°C and 5°C/minute to -100°C. The cryotubes were then stored in liquid nitrogen-filled tanks.

2.1.5 Thawing Cryopreserved Cells

Cryotubes containing frozen cells were rapidly thawed in a 37°C water-bath. The cell suspension was transferred to a 10 ml siliconized glass tube and an equal volume of medium/10% FBS added dropwise, mixed and left to stand for 7 - 10 minutes. An equal volume of medium/10% FBS was again added dropwise to the cell suspension, mixed and left to stand for a further 7 - 10 minutes. The tube was then topped up with medium/10% FBS and centrifuged at 200 g for 5 minutes. The cells were washed two more times to remove all the DMSO.

2.2 IMMUNOASSAYS

2.2.1 Antibody Details

Antibodies against murine c-Kit included a rabbit polyclonal antibody which was a gift from Dr P. Besmer (Cornell University Graduate School of Medical Sciences, New York, NY) and a purified rat monoclonal antibody (mAb) (IgG_{2b}), ACK2, kindly donated by Dr. M. Ogawa (Department of Pathology, Institute for Medical Immunology, Kumamoto University Medical School, Japan) (Ogawa *et al.* 1991). Mouse mAb against human c-Kit included: YB5.B8 (IgG_1) (Gadd & Ashman, 1985), IDC3 (IgG_1) (Aylett *et al.*, 1995) both produced in our laboratory; 17F11 (IgM) (Bühring et al., 1991) which was a gift from Dr. H-J. Bühring (Transplantation Immunology and Immunohematology, University of Tübingen, Germany) and SR-1 (IgG_{2a}) which was generously provided by Dr. V. Broudy (Division of Hematology, Department of Medicine, University of Washington, Seattle). Isotype-matched negative control mAb against *Salmonella* which were produced in our laboratory (O'Connor & Ashman, 1982) included: 3D3.3 (IgG_1), 1D4.5 (IgG_{2a}) and 1A6.12 (IgM). The mAb against murine CD4 (α-L3T4)(IgG_{2b})(ATCC, Cat. No. TIB 207) was used as a negative control for ACK2. Purified α-CD4 was obtained by running hybridoma culture supernatant supplied by Edward Bertram (Department of Microbiology & Immunology, The University of Adelaide, Australia) through a protein G column (provided by Ray Wilkinson, Immuno-Virology, Vetlab, IMVS, Adelaide). The activity of purified α-CD4 was confirmed

through an indirect immunofluoresence assay (see following section 2.2.2) performed on mouse thymocytes. The polyclonal rabbit antiserum to murine GM-CSF and casein were gifts from Dr J. Delamarter (Glaxo).

2.2.2 Indirect Immunofluorescence Assay

Target cells were harvested and washed twice in ice cold PBS containing 0.1% bovine serum albumin and 0.1% sodium azide (PBS-BSA-Az) (latter components were supplemented to PBS after preparation as described in Section 2.1.1). These cells were then resuspended at 10^7 cells/ml in PBS-BSA-Az supplemented with 10% heat inactivated normal rabbit serum (NRS) for assays performed with mouse antibodies or 10% heat inactivated normal sheep serum (NSS) for rabbit antibodies in order to block the binding of Fc receptors, especially FcγRI. Aliquots of 50 µl were dispensed into round-bottomed plastic tubes (Techno-Plas, Cat. No. P-7512, S.A., Australia) and were placed on ice. Saturating levels antibody were added (50 µl) to the cell suspensions, mixed by vortexing and then incubated for 30 - 60 minutes at 4°C. In some assays target cells were incubated with biotinylated-hSLF instead of Ab (as described in Chapter 6) (provided by Mr. Steve Cole, Department of Haematology, Hanson Centre for Cancer Research, Adelaide, Australia).

In situations when cell viability was less than 80% a 10 µl aliquot of ethidium monoazide (EMA) (Molecular probes, Cat. No. E-1374) (100 µg/ml in PBS) solution was added to each tube prior to the above incubation. Cells treated with EMA were incubated on ice in the dark for the first 30 minutes and then placed directly under a fluorescent light for a further 30 minutes, to stimulate the EMA. EMA permeates the cell membrane of dead cells and associates with the DNA thus delineating dead from live cells. The cells were then washed three times in 1 ml of cold PBS-BSA-Az. With each washing the cells were centrifuged at 200 g in a Mistral 3000i, MSE and after the last wash the supernatant was aspirated such that approximately 50 µl of PBS-BSA-Az was remaining. The tubes were then vortexed and to the cell suspension was added either 50 µl of 1:50 dilution (in PBS-BSA-Az/10% NRS or NSS) of affinity-isolated fluorescein isothiocyanate (FITC)-labelled $F(ab')_2$ sheep antibody to mouse Ig (Silenus, Australia, Cat. No. DDAF) or

1:50 dilution of goat α-mouse IgG-R-phycoerythrin (PE) (Southern Biotechnology Associates, Inc. Birmingham, USA Cat No. 1030-09) or 1:10 FITC-labelled $F(ab')_2$ sheep antibody to rabbit Ig (Silenus, Australia, Cat. No. RDAF). Detection of biotinylated-hSLF was performed using streptavidin R-PE (Caltag Laboratories, San Fransisco, CA. Cat. No. SA 1004-4). The tubes were vortexed and incubated at 4°C for 45 minutes in the dark. The cells were washed twice as above, and fixed in 0.5 - 1 ml of cold 1% w/v paraformaldehyde (BDH, Cat. No. 29447) in PBS. The samples were stored, for up to three weeks, at 4°C in the dark until ready to analyse by flow cytometry on a Profile II flow cytometer (Coulter, Hialeah, FL). To ensure reproducibility of mean fluorescence intensity (mfi) values on the flow cytometer from day to day and sample to sample, FL1 (detecting FITC) and FL2 (detecting R-PE) photomultiplier tube sensitivities were standardised using Standard-Brite™ calibration beads (Coulter, Hialeah, FL, Cat. No.PN 6604146).

2.2.3 Fluorescence - activated cell sorting

Cells were stained as above (section 2.2.2) in the absence of sodium azide under sterile conditions and resuspended at approximately 1 - 2 x 10^7cells/ml with ice cold PBS-BSA. Cells were run through the FACSTAR^{plus} cell sorter (Becton-Dickinson, Mountainview, CA) and cells expressing various levels of c-Kit surface antigen were collected. These cells were either cultured in flasks, or for the purpose of clonal isolation, the cells were plated out via limiting dilution in 96 well tissue culture flat-bottomed plates (Nunc, Denmark, Cat. No. 167008).

2.2.4 Quantitation of cell surface human c-Kit

Cell surface c-Kit was determined by using QIFIKIT® microspheres (10 μm diameter) (Biocytex, Marseille, France) essentially as recommended by the manufacturer. Target cells were prepared and incubated with purified mAb, 1DC3 and YB5.B8 (10 μg/ml) or SR-1 ascites (1/20,000 dilution) or their isotype-matched negative controls as described in sections 2.2.1 and 2.2.2. At the end of the primary mAb incubation, 50 μl aliquots of the series of IgG coated microspheres were introduced for the subsequent washes and incubation with 1:50

dilution of PE as described in section 2.2.2 above. The beads, A-G, were coated with 0, 5, 500, 14,000, 29,000, 59,000, 110,000 or 210,000 copies of the murine mAb ST1 (IgG_{2a}-anti-CD5), independently. After the final wash, microspheres and cells were analysed simultaneously on the Profile II flow cytometer using standardised settings.

The mfi of each bead was corrected by subtracting the mfi of bead 'A' (0 copies of ST1) and plotted against the copy number of each bead. Linear regression analysis was then performed using the Microsoft Excel version 5 statistical package. Correlation coefficient was always greater than 0.98. The mfi of target cells was then corrected against the appropriate negative control mAb-treated target cells, and this value was placed into the linear regression formula to calculate the average number of c-Kit molecules on the cell surface.

2.2.5 Alkaline phosphatase anti-alkaline phosphatase (APAAP) technique

Cell smears were made by harvesting cells from culture, washing once in medium/10% FBS, resuspending at 5 x 10^5 cells/ml in medium/50% FBS and 100 µl aliquots centrifuged at 28 g at room temperature (RT) onto ethanol-cleaned microscope slides (Sail Brand, China, Cat. No. 7015) in a Cytospin 3 centrifuge (Shandon Scientific Ltd., England). Cell smears were allowed to air dry before being stored at 4°C in air tight slide boxes in the presence of self-indicating 2 - 4 mm silica gel (Ajax Chemicals, Australia, Cat. No. 3681) until ready to use.

The APAAP technique used for staining of cell smears for the detection of specific antigen expression was a derivation of that described by Erber *et al.*, (1984). The slide box was allowed to warm to room temperature prior to removing slides. A circle was drawn around the cell smear with a Dakopen (Dakopatts, Denmark, Cat. No. S2002) to enable localisation of applied solutions to the cells. Smears were then fixed in standard fixative (see Appendix A1.1.1 for preparation) for 30 seconds. Smears were rinsed in distilled water and then washed in 3 changes of Tris-buffered saline (TBS) (see Appendix A1.1.2 for preparation).

Diluted primary mAb supernatant (30 µl) (1:2 with 10% NRS in PBS-BSA-Az) was applied to the smear and incubated for at least 30 minutes in a humidified chamber at RT to

ensure that the smears did not dry at any stage. Slides could be incubated overnight with primary antibody at 4°C. Smears were then washed in 3 changes of TBS over 5 minutes and 20 µl of diluted rabbit anti-mouse Ig (1:50 with 25% heat inactivated normal human serum in TBS) (Dakopatts, Denmark, Cat. No. Z259) was applied and incubated for 30 minutes at RT and then washed as described above. A volume of 20 µl of diluted APAAP mouse monoclonal complex (1:100 in TBS) (Dakopatts, Denmark, Cat. No. D651) was then applied and incubated for a further 30 minutes at RT prior to washing as above. Smears were then incubated with two further cycles of rabbit anti-mouse Ig/wash/APAAP Ab/wash, with 10 minute Ab incubations. After the final wash the slides were then incubated for 20 minutes at RT in a coplin jar containing the substrate which had been prepared as described in the Appendix A1.1.3. Slides were then rinsed in water and counterstained with haematoxylin (see Appendix A1.1.4) for 1 minute (time dependent on strength), rinsed in tap water, placed in 0.5% HCl in water for 5 seconds, rinsed in water for 10 seconds and then placed in Scott's gentle alkaline solution (see Appendix A1.1.5 for preparation) for 2 minutes. The cells were checked for sufficient counterstaining, and if further staining was required the procedure was repeated from the haematoxylin staining stage. If cells were counterstained sufficiently the slides were rinsed in water and mounted in glycerol-glycine (see Appendix A1.1.6 for preparation). Cells exhibiting antibody binding were stained bright reddish-pink.

2.2.6 Immune rosetting

(a) Purification of red blood cells (RBC) from whole blood

A volume of 5 ml of fresh human heparinized peripheral blood was overlayed onto 3.5 ml of Ficoll-Paque gradient, specific gravity 1.076 (Pharmacia, Sweden, Cat. No. 17-0840-02) in 10 ml polystyrene tubes (Disposable Products, Cat. No. 24221). The Ficoll-Paque and blood were allowed to reach RT prior to use. The tubes were then centrifuged at 400 g for 25 minutes at RT in a Mistral 3000i centrifuge. This procedure separated the red blood cells (RBC) from the granulocytes and mononuclear cells (MNC). The RBC were washed 3 times in Alsever's solution (see Appendix A1.2.1 for preparation) making sure the residual granulocytes were removed each time. After the final wash approximately 2.5 ml of RBC

remained and was made up to 10 ml with Alsever's solution (this suspension could be kept for up to a week at 4°C prior to coupling). A 2 ml aliquot of the RBC suspension was washed 5 times in saline (pH 5 - 6 see Appendix A1.2.2 for preparation) and after the last wash were made up to a 50% solution in saline.

The coupling procedure was a derivation of that described by Parish & McKenzie (1978). A 200 µl aliquot of 50% washed RBC were placed in a 15 ml polystyrene Lux tube (Miles Scientific, Cat. No. 4106) to which was added 1 ml of sterile, affinity-purified goat antibody directed against mouse immunoglobulin (GαM) (provided by S.Cole, Haematology Dept. Hanson Centre for Cancer Research, IMVS, Adelaide, Australia) (approximately 370 - 400 µg/ml, dialysed in saline at a pH of 5-6). This was mixed briefly by vortexing and 1 ml of sterile 'aged' 0.003125% chromic chloride solution ($CrCl_3$) (prepared as described by Goding (1976)) was immediately added dropwise whilst vortexing vigorously. The mixture was allowed to stand for 5 minutes at RT to allow coupling of the immunoglobulin to the RBC. The coupled RBC were washed once in TC-PBS (see section 2.1.1 for preparation) and made up to a volume of 10 ml in TC-PBS. A 1.3 ml aliquot was removed to be used in a haemagglutination assay (as described below) to check for successful coupling. The remaining coupled RBC were made up to 10 ml again and washed as above. A 2% solution of coupled RBC was made by resuspending the washed RBC in 5 ml TC-PBS and stored at 4°C for up to 7 days prior to use.

(b) Haemagglutination Assay

To check the efficiency of the coupling reaction a haemagglutination assay was performed involving the titration of rabbit antibody against goat immunoglobulin (RαG) (3.3 mg/ml stock (Ig2-082/1), a gift from Dr. Peter Ey, Department of Microbiology and Immunology, The University of Adelaide, Australia). 50 µl of a 1:100 and a 1:1000 dilution of RαG in PBS was titrated 1:2 in a round bottomed well microtitre tray (Linbro, Cat. No. 76-042-05). 50 µl of 1% coupled RBC were added to the wells. The tray was left at RT for 2 hours and checked for agglutination. The expected titre for successful coupling of the RBC was approximately 16,000.

(c) Screening of transfectants via immune rosetting

NIH-3T3 cells transfected with pGC1.2 were screened for surface expression of the human c-Kit receptor by immune rosetting. Initially 5 cm diameter dishes containing approximately 20 isolated colonies were washed once with TC-PBS prior to an incubation involving 2 ml of sterile mAb culture supernatants of either 1DC3 and/or YB5.B8 per dish (at least 1 μg/ml/mAb). This incubation was for 1 hour at 37°C in a 5% $C0_2$ atmosphere. Unbound mAb was removed by aspirating the mAb solution from the dishes and rinsing 3 times with TC-PBS. GαM coupled-RBC prepared as described in section 2.2.5 (a) were diluted to a 0.2% solution in DMEM and 2 ml added to each dish and incubated for 1 hour at 37°C in a 5% $C0_2$ atmosphere, with a gentle swirling of the dishes after 30 minutes of incubation. The RBC solution was aspirated and the dishes were rinsed 2 times with TC-PBS prior to scoring RBC-coated colonies under an Olympus microscope.

2.3 PROLIFERATION ASSAYS

Cells from log-phase cultures that had been supplemented with cytokines, as indicated in the results, were washed 3 times in DMEM/10% FBS and resuspended at 2.2 x 10^4 cells/ml. Cell viablity was determined by trypan blue exclusion using a haemocytometer, which was also used to determine cell densities. Aliquots of 450 μl (1 x 10^4 cells) were deposited in duplicate into the wells of 24-well plates (Nunc, Denmark, Cat. No. 1-43982) and varying concentrations of factors were added to a final volume of 500 μl. Cells were incubated for 4 days at 37°C in a humidified atmosphere of 5% CO_2 in air prior to determining cell densities as described above.

2.3.1 ^{3}H-thymidine incorporation assays

In some experiments, as indicated, cell proliferation was determined by ^{3}H-thymidine incorporation. Cells, cultured and washed as above, were resuspended at 10^5 cells per ml and 100 μl aliquots (1 x 10^4 cells) were plated in quadruplicate into 96-well flat-bottomed microtiter trays (Nunc, Denmark, Cat. No. 167008) and factors added to a final volume of 200 μl. Cultures were incubated for 2 days at 37°C prior to being pulsed with

1 μCi ^{3}H-thymidine (specific activity 20 to 40 Ci/mM) (ICN, Cat. No. 2406605) for 18 hours at 37°C. The cells were then harvested onto filter paper (ICN, Cat. No. 78-115-05) and immersed in Biodegradable Counting Scintillant (Amersham, USA. Cat. No. NBCS104) and levels of β-particle emission were determined using a Beckman LS 6000TA scintillation counter.

2.3.2 FDC-P1 co-cultures on fibroblast "feeder" layers

These assays were performed by Dr T. Gonda (Ludwig Institute for Cancer Research, Melbourne, Victoria, Australia). FDC-P1 cells were washed twice, and 2 x 10^4 cells were seeded onto fibroblast monolayers (5 x 10^4 cells) in 1 ml of DMEM containing 10% FBS and polyclonal rabbit antiserum to murine GM-CSF or to casein (1:250 in DMEM/10% FBS) in a 24 well tissue culture plate. Viable cell numbers were determined on days 4 and 7. On day 4, 400 - 500 μl of DMEM/10% FBS containing additional antiserum was added to those wells required on day 7.

Assays for contact-dependence were set up essentially as described above with or without antisera in 1 ml cultures, however, 500 μl of these cultures comprised of 0.5% agar containing DMEM/10% FBS which was overlaid on the fibroblast monolayer to prevent contact with the FDC-P1 cells.

2.3.3 Cell cycle analysis

An aliquot of 10^6 cells was fixed in 1 ml 0.5% paraformaldehyde (in PBS) for 30 minutes at 4°C. The cells were then washed once in 0.1% Triton X100 (Labchem, Ajax Chemicals, Sydney, Australia, Cat. No. 1552) made in PBS-Az (TPBA), resuspended in 1 ml TPBA and then incubated on ice for 3 minutes. Then 25 μg ribonuclease A (RNase A) (Boehringher Mannheim, Germany, Cat. No. 109169) was added and the suspension was incubated at 37°C for 20 minutes. The suspension was then washed once in TPBA and resuspendend in 500 μl propidium iodide (PI) (Sigma, USA, Cat. No. P 4170) at 50 μg/ml in PBS-Az. The tubes were stored in the dark at 4°C until analysed on a Profile II flow cytometer

at least 1 hour after staining. The percentage of cells in Go, G1, M and S phase were calculated from the histograms generated.

2.4 DNA MANIPULATIONS

2.4.1 Restriction Endonuclease Digestion

DNA at a concentration of 0.1 - 4 μg was digested in the presence of 2 - 10 Units of restriction endonuclease according to the method recommended by the manufacturer in a total volume of 20 - 50 μl. Digests were incubated for 1 - 3 hours and the reaction was terminated by heating at 65°C for 10 minutes. If subsequent restriction endonuclease digestions were to be carried out the DNA was phenol/chloroform extracted (procedure described in section 2.4.3(d)) prior to setting up a new reaction.

2.4.2 Analytical and preparative separation of DNA fragments

DNA digests were mixed with loading buffer (prepared as described in A1.7.2. at a final dilution of 1:10. Electrophoresis of digested DNA was carried out at RT in 0.8%, 1%, 1.5% or 2% (w/v) agarose gels, depending on the size of the DNA fragments to be fractionated, in a horizontal gel apparatus. Gels were run at 100 V for 2 - 3 hours in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The gels were stained in ethidium bromide (Sigma, USA. Cat No.E-8751)(2 μg/ml in H_2O) for 5 minutes and then destained in H_2O for 10 minutes whilst being gently rocked. 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.

For preparative gels 1% SeaPlaque GTG (FMC BioProducts, USA, Cat. No. 50111) low gelling temperature agarose was used for separation of DNA fragments. DNA bands to be excised were not exposed to UV but a control track was loaded on the gel which was removed from the remainder of the gel and stained with ethidium bromide. The band was excised from the gel and this lane was then aligned with the remainder of the gel. The corresponding area

was excised from the unstained gel and purified according to one of the following methods depending on the size of the fragment to be purified.

2.4.3 Purification of DNA from agarose or solution

(a) GENECLEAN®

This method was carried out essentially as described by the manufacturer (BIO 101, Cat. No. 3105) and was used to purify DNA of between 0.5 and 3 kb from agarose gels or to purify DNA between enzymatic reactions. The excised gel slice containing the DNA fragment was placed into a 1.5 ml reaction tube and the gel slice weighed. 2.5 - 3 volumes of sodium iodide (NaI) stock solution (provided by the manufacturer) were added and the agarose melted at 55°C in a heat block. To this was added 5 µl of a Glassmilk suspension (provided by the manufacturer), which had been well resuspended by vortexing the tube upside down. The solution was then mixed 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 and the NaI supernatant was removed. The pellet was resuspended and washed 3 times (5 second microcentrifuge spins) with 700 µl of ice cold "NEW WASH" solution (provided by the manufacturer). After the last wash the tube was spun again to remove residual "NEW WASH" and the tube was placed in a heat block for a few minutes at 55°C with the cap off to allow desiccation. The DNA was eluted from the glassmilk with 10 - 20 µl (depending on amount of DNA) TE or 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) Magic Polymerase Chain reaction (PCR) Preps DNA Purification System

This method was carried out essentially as described by the manufacturer (Promega, USA, Cat. No. A7170) and was used to purify DNA fragments of between 500 and 1500 bp in size. The excised gel slice was weighed in a 1.5 ml reaction tube and the agarose was dissolved by heating at 70°C in a heat block. When the agarose was completely dissolved 1 ml of Magic PCR Preps Resin (Cat. No. A7181) was added and the solution vortexed for 20 seconds. A Magic Minicolumn was set up by attaching a minicolumn (Cat. No. A7211) to a

3 ml disposable syringe barrel with the plunger removed. To this was added the DNA/Resin mix and the slurry was gently pushed into the minicolumn with the plunger. The minicolumn was detached from the syringe and the plunger removed. The syringe barrel was reattached to the minicolumn and 2 ml of 80% isopropanol was washed through the column by pushing it through the minicolumn with the plunger. The syringe was removed and the minicolumn was transferred to a 1.5 ml reaction tube and microfuged for 20 seconds to dry the resin. To remove any residual moisture from the resin the minicolumn was placed in a Speedvac (Savant) for 2 minutes. The minicolumn was transferred to a new tube and the DNA eluted from the resin by adding 50 µl of TE or H_2O and allowing it to stand for 5 minutes prior to microfuging for 20 seconds.

(c) β-Agarase 1 Treatment

This method was used to purify DNA fragments greater than 3 kb or when purifying low amounts of DNA contained in agarose and was performed according to the protocol supplied by the manufacturer with minor modifications. The gel slice (approximately 200 µl in volume) was melted by adding 1/10 volume of 10 x buffer (New England BioLabs, Cat. No. 392S) and heating at 65°C for 10 minutes, mixing every 2 - 3 minutes. The solution was chilled to 40°C by placing the heat block in ice for a few seconds and to this was added 2 units/200 µl of β-agarase 1 enzyme (New England BioLabs, Cat. No. 392S). This was incubated overnight at 40°C, chilled on ice for 15 minutes and then microfuged for 15 minutes at 4°C to remove any carbohydrates. The supernatant was removed and 1/10 volume of 3 M sodium acetate (NaAc) pH 4.6 along with 1 µl glycogen (of a 20 mg/ml stock) (Boehringher Mannheim, Germany, Cat. No. 901 393) and 2.5 volumes absolute ethanol were added and the DNA precipitated for 30 minutes on ice. The DNA was pelleted by microcentrifuging for 30 minutes at 4°C and the pellet washed twice in 70% ethanol. The pellet was dried under vacuum and dissolved in 10 µl TE.

(d) Phenol extraction

Between DNA manipulations DNA was purified by phenol/chloroform back extraction. The volume of the DNA solution was made up to a minimum of 100 µl with sterile distilled H_2O containing 5 µg glycogen (Boehringer Mannheim, GmbH., Germany. Cat. No. 901 393) Then an equal volume of phenol/chloroform (1:1) was added. This was mixed well and incubated on ice for 3 minutes and then spun for 3 minutes in a microcentrifuge. Two thirds of the aqueous layer was removed and replaced with an equal volume of H_2O this was then mixed and incubated on ice for a further 3 minutes prior to microfuging and the aqueous phase collected as before. This was repeated once more and 1/10 volume NaAc, pH 4.6 and 2 volumes absolute ethanol added to the DNA solution. The DNA was precipitated by incubation on ice for 30 minutes and microfuging for 15 minutes at 4°C. The pellet was washed in 70% ethanol, dried and dissolved in H_2O or TE.

2.4.4 Size determination of DNA fragments

The sizes of restriction fragments were calculated 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, Cat. No. DMW-L1), *Eco*R1 digested *Bacillus subtillis* phage SPP1 DNA (Bresatec, Adelaide, Cat. No. DMW-S1) and *Hpa* II digested plasmid pUC19 DNA (Bresatec, Adelaide, Cat. No. DMW-P1). The estimated sizes of these molecular weight markers in kilobases are as follows:

Hind III digested λ DNA: 23.1; 9.4; 6.6; 4.37; 2.3; 2.0; 0.564; 0.125

Hpa II digested pUC19 DNA: 0.501; 0.489; 0.404; 0.331; 0.242; 0.190; 0.147; 0.111; 0.110; 0.067; 0.034; 0.026

SPP1: 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

2.4.5 Quantitation of DNA

The concentrations of DNA in solution were determined either by measuring the absorption at 260 nm on a spectrophotometer, assuming that an $A_{260}1 = 50$ μg/ml of DNA or by electrophoresis of DNA in agarose gels and comparing the intensities of ethidium bromide - stained bands with the intensities of bands containing known concentrations of DNA standards.

2.4.6 End-filling DNA fragments

End-filling of DNA to create blunt ended fragments was carried out essentially as described by Sambrook *et al.*, (1989). DNA was incubated with 1 - 2 units/μg of Klenow polymerase (Pharmacia, Sweden, Cat. No. 27-0928-01) and a solution of 2'-deoxynucleotide 5' triphosphate (dNTP) (containing 2 mM each of dATP, dCTP, dGTP, dTTP) (Pharmacia, Sweden. Cat. No. 27-20(5-8)0-02) was added such that the final concentration of each dNTP was 1 mM in a reaction volume of 20 μl. This was incubated at room temperature for 30 minutes. For DNA which had been gel purified TE (1 x final) and $MgCl_2$ (5 mM final) were also added to the above reaction in a final volume of 20 μl. Klenow polymerase was inactivated by heating at 75°C for 10 minutes. DNA was not purified if it was to be used in ligation reactions (as described in section 2.4.9) but if subsequent enzymatic reactions were to be carried out DNA was purified by phenol extraction (see section 2.4.3(d)).

2.4.7 Dephosphorylation of DNA

Restriction digested and end-filled plasmid DNA was dephosphorylated by using calf intestinal alkaline phosphatase (CIP) (Boehringher Manneheim, Germany, Cat. No. 713 023) essentially by the method described by Sambrook *et al.*, (1989) and Dr. C. Hutchins (personal communication; Leukaemia Research Unit, Hanson Centre for Cancer Research, Adelaide, Australia). Digested plasmid DNA (1 μg) was incubated with 1 unit of CIP/2 pMoles of 5' termini in 1 x CIP dephosphorylation buffer (10 x CIP Buffer: 10 mM $ZnCl_2$, 10 mM $MgCl_2$, 100 mM Tris-HCl pH 8.3. This buffer remains as a precipitate and requires vortexing

prior to use. A duplicate reaction was also set up containing a 10 fold higher concentration of CIP. If necessary, CIP was diluted in 1 x CIP dephosphorylation buffer. The reaction was incubated at 37°C for 15 minutes and then another aliquot of CIP was added and the reaction was incubated at 55°C for 45 minutes for blunt ended DNA. 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. To test that the ends had been dephosphorylated successfully, ligation reactions (see section below 2.7.8) including both CIP treated (various concentrations) and untreated vector were performed. These reactions were introduced into E.*coli* as described in section 2.4.10 along with unligated CIP treated vector and the number of transformants were compared. This enabled background colony numbers to be determined prior to ligating the dephosphorylated vector with the insert DNA. The lowest amount of CIP used that gave efficient dephosphorylation was chosen for use in subsequent ligations.

2.4.8 Kinasing

DNA 5' ends were phosphorylated using T4 polynucleotide kinase (PNK) (10 U) (Pharmacia, Sweden, Cat no 27-0736-01) in the presence of 66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.1 mM spermidine and 1 mM rATP (Pharmacia, Sweden, Cat. No.27-2056-01), pH 7.4 in a final reaction volume of 10 µl at 37°C for 30 minutes. PNK was then inactivated by heating at 65°C for 10 minutes.

2.4.9 Ligation

Ligation reactions contained 0.5 - 50 ng of vector DNA and a 1 - 3 fold molar excess of insert DNA in the presence of 25 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.4 mM ATP, pH 7.4 (or in kinase buffer above) and 2 Weiss Units of ligase (Progen, Australia, Cat. No. 700-0000), in a final reaction volume of 10 µl. The ligation reaction was incubated overnight at 16°C in a Frigomix 1497 (B.Braun). The ligase was then inactivated by heating at 65°C for 15 minutes and allowing to cool at RT prior to all or a fraction of the ligation reaction being transformed into E.*coli* (see sections 2.4.11 and 2.4.12).

2.4.10 Production of competent bacterial cells

(a) Calcium chloride competent cells

E.*coli* DH10β strains (BRL, Cat. No. 8297SA) were made competent for transformation by a procedure similar to that described by Sambrook *et al.*, (1989). Cells were streaked from glycerol stocks on to Ψa agar plates (see Appendix A1.4.1) and left to grow overnight at 37°C. A single colony was used to innoculate 5 ml Ψb medium (Appendix A1.4.2) and this was incubated with shaking for 2 hours or until the A_{650} = 0.6. This was subcultured 1:20 into 100 ml Ψb medium (prewarmed at 37°C) and grown for a further 2 hours or until A_{650} = 0.6 - 0.8. The culture was chilled on ice for 5 minutes and spun in a Beckman J-21C Centrifuge in a JA14 rotor at 3,550g for 5 minutes at 4°C. The pelleted cells were resuspended in 40 ml of cold Tfb I Buffer (see Appendix A1.4.3). This was allowed to stand on ice for 5 minutes prior to pelleting the cells again by centrifuging at 3,550g for 5 minutes at 4°C. The pellet was resuspended in 4 ml of Tfb II Buffer (see Appendix A1.4.4) and left on ice for 15 minutes prior to aliquoting 200 µl into cold 1.5 ml reaction tubes on dry ice using a pre-chilled tip. The cells were snap frozen in dry ice and stored at -70°C.

(b) Electrocompetent cells

E.*coli* DH10β and MC1061 strains were made electrocompetent by the procedure derived from the protocol published by Dower *et al.* (1988). These cells were used in preference to those produced by the method described in 2.4.10 (a) when a high transformation efficiency was required. Cells were made electrocompetent by inoculating a 10 ml "superbroth" (see Appendix A1.4.5) with an isolated colony and growing the culture overnight at 37°C with shaking. This was subcultured into 1 litre of prewarmed "superbroth" and grown until the OD_{600} = 0.4 - 0.6. The culture was chilled on ice prior to pelleting at 2,500g for 15 minutes using a Beckman J-21C centrifuge and a JA14 rotor. The pellet was washed 3 times with 250 ml ice cold sterile H_2O and the final pellet resuspended in 5 ml of cold 10% glycerol. The solution was pooled and the cells pelleted at 3,000g for 15 minutes using a Beckman J-21C centrifuge and a JA20 rotor at 4°C. The pellet was resuspended in

1 ml 10 % glycerol and the cells aliquoted into cold 1.5 ml reaction tubes, snap frozen on dry ice and stored at -70°C. The transformation efficiency of the cells was tested prior to use by transforming 10 pg of pUC19 DNA (as described in section 2.4.12 below) and the efficiency determined as follows:

$$\text{transformation efficiency(CFU/}\mu\text{g)} = \frac{\text{CFU in control plate}}{\text{pg pUC19 used}} \times \frac{10^6 \text{pg}}{\mu\text{g}} \times \text{dilution factor(s)}$$

2.4.11 Transformation of $CaCl_2$ competent *E.coli.*

Competent cells that were prepared as described in section 2.4.10 (a) stored at -70°C were allowed to thaw on ice and 100 µl was placed in a cold 10 ml polypropylene tube (Disposable Products, Australia, S.A., Cat. No. 24268). To the cells a maximum of 10 ng of DNA from the ligation reaction was added and the cell/DNA mix was incubated on ice for 30 minutes. The cells were heat-shocked for 45 seconds in a 42°C waterbath and immediately placed on ice for 2 minutes prior to adding 0.9 ml of S.O.C. medium (see Appendix A1.4.6). The cells were incubated at 37°C with shaking for 1 hour. An 100 µl aliquot of neat, 1:10 and 1:100 dilutions of the reaction mix were spread onto Luria Broth (LB) plates (see appendix A1.4.9) containing 100 µg/ml ampicillin (Sigma, USA, Cat. No. A-9518).

2.4.12 Transformation of Electrocompetent E.*coli*

Electrocompetent cells that were prepared as described in section 2.4.10 (b) were thawed at room temperature. The ligation reaction to be transformed was phenol/chloroform extracted as described in section 2.4.3(d) and washed twice with 70% ethanol prior to drying under vacuum and redissolving the pellet in sterile distilled 2 µl of H_2O. Alternatively, depending on the concentration of DNA in the ligation reaction, the latter was diluted 10 fold and 2 µl used for transformation. The DNA was placed in a 1.5 ml reaction tube on ice along with 0.1 cm cuvettes (Biorad, Cat. No. 165-2089). To the DNA was added 40 µl of thawed electrocompetent cells, which were mixed by pipetting with a cold tip. This was left to stand for 1 minute on ice prior to transferring to a cold cuvette and electroporating at 200 Ω, 1.6 kV,

25 μF with a Bio-rad Gene Pulser. A time constant of 4.7 μsec was obtained. Immediately after electroporation 1 ml S.O.C. medium (Appendix A1.4.6) was added to the cuvettes using a glass pasteur pipette and then transferred to a 10 ml polypropylene tube and incubated at 37°C with shaking for 1 hour. Neat and diluted aliquots (100 μl) of the transformation culture were then plated on LB plates (see Appendix A1.4.8) containing 100 μg/ml ampicillin.

2.4.13 Small scale plasmid preparations

This method of plasmid extraction is a modification of the boiling method described by Sambrook *et al.*, 1989. A single colony was isolated and used to innoculate 2 - 5 ml of LB (see Appendix A1.4.7) overnight at 37°C with shaking. The culture was chilled on ice prior to spinning 1.5 ml in a 1.5 ml reaction tube for 1 minute at 4°C in a microcentrifuge. The supernatant was aspirated and the pellet resuspended in 250 μl of freshly prepared TELT buffer (50 mM Tris.HCl pH 7.5, 62.5 mM EDTA, pH 8, 0.4% Triton X100 and 2.5 M LiCl). This was vortexed vigorously and left on ice for greater than 5 minutes after which 20 μl of freshly prepared lysozyme (10 mg/ml) was added. The mixture was vortexed for 3 seconds and then boiled for 1 minute and put immediately on ice for greater than 15 minutes. The mixture was then spun for 20 minutes at RT and the supernatant transferred to a fresh tube. The DNA was precipitated by adding 500 μl of absolute ethanol to the lysate and vortexing the solution prior to microfuging for 15 minutes at 4°C. The pellet was washed in 70% ethanol, dried under vacuum and resuspended in 50 μl of TE pH 8.0 or H_2O. If the plasmid DNA was to be used for sequencing (see section 2.6) the pellet was resuspended in 32 μl of T.E. pH 8. If the plasmid preparation was performed to verify whether E.*coli* transformants had taken up plasmid DNA restriction endonuclease digestion and subsequent electrophoresis were performed as described in sections 2.4.1 and 2.4.2 and positive transformants were chosen to produce glycerol stocks. Glycerol stocks were produced by streaking the original positive transformant colony onto LB agar plates containing ampicillin and incubating overnight at 37°C. The bacterial lawn was then harvested and stored in 1 ml of a 1% peptone, 20% glycerol solution at -20°C. The original colony could also be used to inoculate a 10 ml culture for larger scale plasmid preparations as described below.

2.4.14 Midiprep DNA method

This method of plasmid DNA preparation was a modification of the alkali lysis method described by Sambrook *et al.*, (1989) and was adequent to use to transfect ψ2 cells (see sections 2.8.3). A 100 ml LB culture supplemented with 100 μg/ml ampicillin was grown overnight. The cells were pelleted at 2,500 g for 5 minutes at 4°C in a Beckman J-21C centrifuge using a JA14 rotor. The pellet was resuspended in a total of 2.5 ml of Solution I (50 mM glucose, 25 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0) for 5 minutes at RT followed by the addition of 5 ml of Solution II (0.2 M NaOH, 1% sodium dodecyl sulphate (SDS); freshly made) and further incubated for 10 minutes. To this was added 3.75 ml Solution III (30 ml 5 M KAc, 5.75 ml glacial acetic acid, 14.25 ml Milli-Q H_2O) and the suspension was incubated on ice for a minimum of 10 minutes. Chromosomal DNA was pelleted at 17,700g in a Beckman J-21C centrifuge using a JA20 rotor for 30 minutes at 4°C. The supernatant was transferred to a 50 ml polypropylene tube (Corning, NY, 25331-50) and to this was added 50 μl of a 10 mg/ml solution of boiled RNase A (Boehringher Mannheim, Germany, Cat. No. 109169). RNA digestion was allowed to occur for 30 minutes at 37°C. The RNase A and residual chromosomal DNA were removed by adding an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and the phases were separated by spinning at 2,000 g for 10 minutes at RT in a Beckman J-6B centrifuge. To the aqueous layer was added 0.6 volume of isopropanol. This was vortexed and the DNA was pelleted at 16,600g in a Beckman J-21C centrifuge using a JA20 rotor for 10 minutes at 4°C. The pellet was washed with 70% ethanol and dried under vacuum prior to resuspending the DNA in 400 μl of sterile distilled H_20 or TE pH 8.

2.4.15 Large scale plasmid preparation

10 ml of LB (see Appendix A1.4.7) containing ampicillin (100 μg/ml) was inoculated with an isolated colony and the culture was grown to log-phase. This was sub-cultured into 500 ml LB (+ ampicillin) and grown overnight at 37°C with shaking. The culture was placed in two 250 ml centrifuge buckets and spun at 1,570g for 20 minutes in a pre-cooled Beckman

J-21C centrifuge using a JA14 rotor. The supernatant was discarded and each pellet resuspended in 10 ml of Plasmid Buffer (25 mM Tris-HCl, pH 8 , 10 mM EDTA, 15% sucrose). To these were added 2 ml of freshly prepared lysozyme solution (25 mg/ml in Plasmid Buffer). The solutions were transferred to two 35 ml tubes and incubated on ice for 40 minutes prior to adding 12 ml of freshly prepared Lysis Buffer (0.2 M NaOH, 1% SDS) to each. The tubes were incubated for 10 minutes on ice and then 7.5 ml of 3 M NaAc, pH 4.6 was added, mixed and further incubated for 40 minutes. Chromosomal DNA was pelleted by centrifuging at 17,700 g in a Beckman J-21C centrifuge using a JA20 rotor for 20 minutes at 4°C. The supernatant was collected carefully and transferred to clean 50 ml polypropylene tubes (Corning, NY, 25331-50) and phenol/chloroform/isoamyl alcohol (25:24:1) extracted 3 times. The phases were separated by spinning at 2000 g for 10 minutes in a Beckman J-6B centrifuge. The upper DNA layers were pooled into large centrifuge buckets and precipitated by adding 2.5 volumes of absolute ethanol and placing at -70°C for 30 minutes (or 4°C overnight). The DNA was pelleted at 5,000 g in a Beckman J-21C centrifuge using a JA20 rotor for 10 minutes and the pellet washed in 70% ethanol. The pooled plasmid DNA obtained from a 500 ml culture was resuspended in 5.9 ml TE pH 8 and purified by centrifugation through a caesium chloride (CsCl) gradient. To the DNA solution was added 6.44g CsCl (BDH, U.K., Cat. No. 810709) and 80.6 µl of 10 mg/ml ethidium bromide. The CsCl was allowed to dissolve and the mixture loaded equally into 2 OptiSeal tubes (Beckman,USA, Cat. No. 361621) using a 5 ml syringe and 18 gauge needle. The tubes were balanced using a 1.1 mg/ml CsCl solution. The tubes were sealed and spun at 337,000 g in a TLA-100.4 rotor in a Beckman Optima TLX ultracentrifuge overnight at 20°C. The DNA band was removed using a 19 gauge needle and 3 ml syringe. The ethidium bromide was removed by extracting several times with 5 M NaCl-TE saturated isopropanol (see appendix A1.4.9). The extracted DNA was placed into sterile dialysis tubing (see Appendix A1.4.10) and dialysed 3 times against 1 x TE pH 8.

2.5 Polymerase Chain Reaction (PCR)

All reagents were aliquoted using non-aerosol tips in order to reduce contamination. Reactions were set up in 0.5 ml micro-centrifuge tubes (Edwards Medical Supplies, NSW, Australia. Cat. No. 3020-500) containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 200 M each of dATP, dTTP, dCTP, dGTP (Pharmacia, Sweden. 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 a final volume of 50 μl for the reaction mix. 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 prior to 25 cycles consisting of: 94°C for 1 minute, 45 - 65°C for 1 minute (depending on the Tm of the oligos; Tm = 4x(G+C) + 2x(A+T)), 72°C for 1-3 minutes (according to the rule that extension occurs at 1 kb/minute) followed by a final extension at 72°C for 7 minutes.

2.5.1 Purification of oligonucleotide primers

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 inserting a 1 ml syringe into one end of the column and into the other end another 1 ml syringe containing 500 μl of ammonium hydroxide (25% ammonia solution, MERCK, Cat. No. 1.05428) was inserted. The plungers of the syringes were moved backwards and forwards in order to fill the column with ammonium hydroxide and left to stand for 20 minutes. The ammonium hydroxide was then drawn into one syringe and the contents placed in a 2 ml screw-capped tube. 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 the primer dried down by vacuum in a Speedvac. The pellet was dissolved in 100 μl of sterile distilled H_2O and the concentration determined by spectrophotometry $A_{260}1$ = 33 μg/ml or to determine molarity the following

formula was used: concentration of oligo(M) = $\frac{x\ \mu g \times 10^{-6} g}{\text{oligo mwt}}$ / 1×10^{-3}

molecular weight (mwt) of dNTPs: A=347.2, T=332.2, G=363.2, C=323.2

If the synthesis of full length oligonucleotide primers was required for site-directed mutagenesis, primers were synthesised with a trityl group on their 5' end. After removal from the synthesis column as described above the primer was subsequently purified by passing through an oligonucleotide purification column (OPC) via the following procedure: 5 ml of acetonitrile (Applied Biosystems, USA, Cat. No. 400060) was passed through a 10 ml syringe barrel attached to the OPC to waste. 5 ml of 2 M Triethylammonium Acetate (TEAA)(Applied Biosystems, USA, Cat. No. 400613) was then passed through to the waste. The oligonucleotide ammonia solution was diluted with an equal volume of H_2O (1 ml) and passed through the OPC 1 drop/second and the eluate was collected and passed through for a second time. Then 5 ml of a 1:10 diluted ammonia solution was passed through the column followed by 10 ml of H_2O to the waste. The syringe barrel was then filled with 5 ml 2% trifluoroacetic acid (TFA) in H_2O (Applied Biosystems, USA, Cat. No. 400445) and a portion of the solution was passed through the column to waste. This was allowed to let stand for 3 - 5 minutes before passing the remainder of the solution through. This was followed by passing another 10 ml of H_2O through prior to eluting the purified primer from the column drop-by-drop with 1 ml of 20% v/v acetonitrile in H_2O. This was collected into a 2 ml eppendorf tube and dried down overnight under vacuum. The primer was resuspended in 100 µl sterile distilled H_2O.

2.5.2 Oligonucleotide Primers

Table 2.5.2.1 summarises the location of the oligonucleotide primers used to sequence or amplify human c-*kit*. The location of each of these oligonucleotide primers is based on the c-*kit* sequence published by Yarden *et al.*, (1987a).

Table 2.5.2.1: Oligonucleotide Primers

name	oligonucleotide sequence*	location	orientation
387	5'-*GGGGGATCC* ATGAGAGGCGCTCGCGGC-3'	22-39	sense
SRC-02	5'-CTGTCTGGACGCGAAGCA-3'	70-87	anti-sense
1032	5'-TGCACCAACAAACACGGC-3'	309-327	sense
1869	5'-GATTCTGGAGTGTTCATG-3'	871-889	sense
1868	5'-CAAGGTTGTTGTGACATTTGC-3'	917-937	anti-sense
764	5'-AGTTCACTTACGTATCTG-3'	1140-1151	anti-sense
765	5'-AGTGAACTTCATCTAACG-3'	1144-1161	sense
1142	5'-CTAGTGGTTCAGAGTTC-3'	1435-1451	sense
447	5'-*GGGGGATCC* GATGTGGGCAAGACTTCT-3'	1506-1524	sense
796	5'-CTTTAAATGCAAAGTTAAAATAGGC-3'	1525-1549	anti-sense
797	5'-AGCAAATCCATCCCCACACC-3'	1562-1581	sense
388	5'-*GGGGGATCC* GGTGTGGGGATGGATTTG-3'	1564-1581	anti-sense
SRC-01	5'-CAGCAAAGGAGTGAACAG-3'	1582-1599	anti-sense
445	5'-*GGGGGATCC* CTGTACTTCATACATGGG-3'	1672-1689	anti-sense
1002	5'-GATAGTACTAATGAGTACATGG-3'	2167-2188	sense
1003	5'-GCAGGAAGACTCCTTTGAATGC-3'	2142-2163	anti-sense
1004	5'-TCATCCTCCATGATGGCG-3'	2283-2300	anti-sense
SRC-04	5'-ATTTTGGTCTAGCCAGAG-3'	2451-2468	sense
SRC-03	5'-TTAGAATCATTCTTGATG-3'	2470-2487	anti-sense
SRC-05	5'-GAATGGTCTACCACGGGC-3'	2865-2882	anti-sense
Universal	5'-*GTAAAACGACGGCCAGT*-3'	-	sense
T3	5'-*ATTAACCCTCACTAAAGG*-3'	-	anti-sense

* sequences in italics do not correspond to c-*kit*

2.6 SEQUENCING

2.6.1 Dideoxy Sequencing of DNA

Sequencing of double-stranded DNA was performed using the Super-Base Sequencing Reagent Kit (Bresatec, Adelaide. Cat. No. SBK-2) essentially following the protocol provided by the manufacturer. The template DNA was prepared as described in section 2.4.12 and was first denatured by alkaline treatment as follows:

DNA	8 - 12 µl (approximately 2µg)
2 M NaOH	4 µl
Water	make up to 20 µl

This was incubated at room temperature for 10 minutes prior to precipitating the DNA by adding:

3 M NaAc, pH 4.5	6 µl
Ethanol	120 µl
Water	14 µl

This was mixed well and incubated at -70°C for 20 minutes. The DNA was pelleted by spinning for 10 minutes in a microcentrifuge and the pellet washed with 70% ethanol. The pellet was vacuum-dried and the primer allowed to anneal to the template by resuspending in:

Water	7 µl
5x annealing buffer	2 µl
primer *	100 ng
	10 µl

* see Table 2.5.2.1 for list and location of primers used

This was mixed well and incubated for 30 minutes at 37°C and then incubated at RT for 20 minutes.

To the annealed primer/template mix were added:

diluted labelling/extension mix*	2 µl
100 mM DTT	1 µl
Water	1 µl
α-^{35} S-dATP(10 µCi)**	1 µl
T7 DNA Polymerase (2 Units)***	1 µl

*The labelling/extension mix was diluted according to the distance of the sequence required to be read after the primer binding site. If a sequence close to the primer was required, for example within 5 - 20 bases, then this mix was diluted 1:15 favouring the generation of short strands of DNA. If longer extension was required 1:10 or 1:5 dilutions were used.

**α -^{35}S-dATP was purchased from Bresatec (Adelaide, Cat No. SDA-2).

*** T7 DNA Polymerase was purchased from Pharmacia (Sweden, Cat. No. 27-0985-04) and was diluted to 2 units/µl in the provided enzyme diluent.

To the wells of a micro tray (Disposable Products, Australia, Cat. No. 239728) were added 2.5 µl 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. After the addition of the enzyme the reaction was incubated for 4 - 5 minutes prior to transferring 3.5 µl 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 used on the same day or stored at -20°C for up to a week. Before loading on to a gel the samples were denatured at 95°C for a few minutes on a heat block and loaded directly onto the gel (see below).

2.6.2 Sequencing Gel

Sequencing gel mix (70 ml) was made by mixing 35 ml of 46% Urea (Appendix A1.5.1), 28 ml of 20% acrylamide (Appendix A1.5.2), 7 ml of 10 x TBE (Appendix A1.5.2), 350 µl of 10% ammonium persulphate (Sigma, USA, A-9164) and 70 µl of TEMED (Sigma, USA, T-8133). This was mixed and immediately taken up into the barrel of a 50 ml syringe. This solution was poured, using a 19 gauge needle, in between two taped and clamped glass

plates (dimensions 42.5 cm x 33.5 and 39.5 x 33.5; the smaller glass plate was coated with Coatasil (Ajax Chemicals, Cat. No. 2293)). The gel was allowed to polymerise for a minimum of 20 minutes prior to removing the comb and setting up for gel electrophoresis. The wells were flushed with TBE buffer to remove unpolymerised acrylamide and pre-electrophoresed for 30 minutes at 1700 - 1800V. The wells were again flushed out prior to loading the denatured samples. The gel was allowed to electrophorese for various periods of time depending on the position of the sequence to be read. If however a longer DNA sequence was required then samples were loaded at staggered time intervals. A usual run involved loading 2 µl of each of the denatured sequence reactions (A,G,C,T) and the gel was electrophoresed until the slower dye front was close to the bottom of the gel. The samples were denatured again and another 2 µl was loaded and allowed to run until the slow dye front was 3/4 of the way down the gel. The third loading was allowed to run until the fast dye front was close to the bottom of the gel.

After electrophoresis the plates were separated and the gel was fixed with 1 litre of a 10% methanol/10% acetic acid solution over a period of 30 minutes. The gel was then transferred to Whatman 3M filter paper and dried under vacuum at 80°C for 40 minutes. The dried gel was autoradiographed overnight at -70°C.

2.7 PREPARATION AND ANALYSIS OF mRNA

2.7.1 Preparation of poly A^+ mRNA

All reagents and apparti used in the preparation of mRNA were made RNase-free as described in Appendix A1.6.1. Poly A^+ mRNA was prepared by the protocol described by Gonda *et al.*, (1982). mRNA was collected from 10^7 - 5 x 10^7 cells. Cells were collected in 50 ml polypropylene tubes (Corning, NY, 25331-50) and washed several times in TC-PBS. Pelleted cells were usually snap frozen in liquid nitrogen and stored at -70°C although mRNA could be extracted from freshly washed cells by resuspending in STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA) to 2 - 5 x 10^6 cells/ml. Predigested proteinase K (MECK, Cat. No. 24568) and SDS were added to a final concentration of 200 µg/ml and 0.5%, respectively. If pellets were snap frozen then STE, proteinase K and SDS were added together

and homogenised immediately for 30 seconds on a medium to high speed ultra-turrax (Omni-5000, USA) which had been made RNase-free as described in Appendix A1.6.2. This was then incubated at 37°C for 1 hr in a water bath. The solution was made up to 0.5 M with respect to NaCl and 2 ml RNase-free treated oligo-dT (Boehringher Mannheim, Germany, Cat. No. 808229) cellulose suspension (see Appendix A1.6.3 for preparation) was added. This was mixed for 1 - 2 hours on a rotator, and the oligo-dT cellulose was spun down at 670 g for 5 minutes in a Mistral 3000i centrifuge. The oligo-dT was resuspended in 3 ml Binding Buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS) and transferred to an alkali-treated poly-prep chromatography column (Bio-Rad, Cat. No. 731-1550). Then 6 ml of Binding Buffer followed by 2 ml of Wash Buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS) were sequentially passed through the column followed by elution of the mRNA from the oligo-dT cellulose with 1.6 ml of Elution Buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1% SDS). To the eluant was added 1/10 volume 0.3 M NaAc, pH 5.6 and 2.5 volumes absolute ethanol. This was stored at -20°C overnight in 2 ml reaction tubes (Eppendorf, Germany, Cat. No. 10030 120.094). The mRNA was precipitated by microfuging for 30 minutes at 4°C. The supernatant was aspirated and the pellet vacuum - dried. The pellet was resuspended in 400 µl TE and centrifuged for 30 seconds at 4°C to remove residual oligo-dT cellulose. A 40 µl aliquot was removed and the concentration of mRNA determined using a spectrophotometer ($A_{260}1$ = 40 µg/ml). To the remainder of the mRNA sample was added 1/10 volume NaAc (pH 5.6) and 2.5 volumes absolute ethanol and was stored at -20°C till ready to use.

2.7.2 Probes

Full length murine c-*kit* cDNA (3 kb), according to sequence published by Qui *et al.* (1988) was excised from pSPK-1 (provided by Dr. T. Gonda, Hanson Centre for Cancer Research, Adelaide, Australia) with the restriction endonucleases Xho 1 - Hind III. Full length human c-*kit* cDNA (3 kb), according to the published sequence of Yarden *et al.* (1987a) was excised from pBluescript (SK^-) (provided by Dr. Douglas Williams, Immunex Corp., Seattle, WA) with the restriction endonucleases Asp 718-Not I. A 780 bp fragment of

the human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) cDNA excised from pHcGAP (ATCC, Rockville, MD) was provided by Mr. Graeme Casey, Department of Haematology, IMVS, Adelaide, Australia. A 540 bp Sca I-Bgl II fragment of the murine dihydrofolate reductase gene (*DHFR*) was excised from the pRSVOO9/A$^+$ vector (see Figure 5.2.1 for diagram of vector). A 2.06 kb Sal I fragment containing the full coding sequence of murine SLF was excised from pBSSK.MGF.10 (Anderson *et al.*, 1990) (provided by Dr. Douglas Williams, Immunex Corp., Seattle, WA). All were digested according to the procedure described in section 2.4.1 and the DNA fragments were size fractionated in 1 - 1.5% low melting point Seaplaque agarose (FMC, USA, Cat. No. 50102) gels. The appropriate fragments were excised and the gel was purified as in section 2.4.3 (a,b).

2.7.3 Random oligonucleotide priming

Double stranded DNA probes were labelled using the GIGAprime DNA Labelling Kit (Bresatec, Adelaide. Cat. No. GPK-1) with minor modifications to their protocol. DNA (50-100 ng in a volume of 1 - 6 µl) was denatured in 6 µl of decanucleotide solution by heating at 95^{o}C for 5 minutes and then placed on ice. To this was added; 6 µl of the appropriate nucleotide/buffer cocktail, according to the isotope(s) to be used; 5 µl (50 µCi) α-^{32}P-dCTP or α-^{32}P-dATP (Bresatec, Adelaide Cat. No. ADC-3 or ADA-3) and 1 µl (5U) Klenow DNA polymerase (provided in the Kit). The final reaction volume was adjusted to 24 µl with H_2O. If small PCR products (ranging in size from 50-100 bp) were to be labelled then specific primers (100 ng each) were used instead of the decanucleotide solution and both α-^{32}P-dCTP and α-^{32}P-dATP were used in the reaction. This was mixed and incubated at 37^{o}C for 30 minutes. For the labelling of probes greater than 100 bp unincorporated label was removed using a MicroSpin™ S-300 HR column (Pharmacia Biotech, USA. Cat. No. 27-5130-1). Prior to adding the probe to the column it was vortexed and microfuged at 735 g for 1 minute to set up the sepharose column. The reaction mix was added dropwise to the column inserted into a 2 ml reaction tube and spun at 735 g for 2 minutes to remove unincorporated label. For probes smaller than 100 bp unincorporated label was removed by adjusting the volume of the reaction to 50 µl with H_2O and then precipitating the

labelled-DNA with 25 µl of 7.5 mM ammonium acetate and 150 µl absolute ethanol for 30 minutes at -20°C. The labelled-DNA was pelleted by microfuging for 30 minutes at RT and the pellet was washed 1 - 2 times with 70% ethanol to remove unincorporated label. The DNA was dried under vacuum and redissolved in 100 µl H_2O. The specific activity of the DNA probe was determined by diluting 1 µl of the probe in 100 µl and placing 5 µl into a Bioscan QC.2000 beta counter. Specific activity (SA) was calculated using the following formula:

SA (total cpm) = counts x conversion factor (5) x dilution factor x volume of probe

Hybridisations were performed in the presence of 10^6 cpm/ml of probe in the presence of an additional 100 µg/ml sheared salmon sperm DNA (see section 2.7.4 below). The volume of labelled probe required was mixed with the salmon sperm DNA and denatured at 100°C for 5 minutes and immediately placed on ice. This was then added to the bag containing the hybridisation solution and filter as described below.

2.7.5 Northern Blot Transfer

Poly A^+ selected mRNA (2 µg) isolated as described in section 2.7.1 and 10 µg of RNA molecular weight markers (Boehringher Mannenheim, Germany. Cat No. 1062 611) were pelleted in the presence of 20 µg glycogen by microfuging at 13,000 g, 4°C, for 15 minutes. The mRNA was washed in 70% ethanol, dried under vacuum and redissolved in 12 µl of sample buffer (see Appendix A1.7.1). The mRNA was denatured by heating at 60°C for 5 minutes. Samples were placed immediately on ice and 3 µl of gel loading buffer (see Apendix A1.7.2) was added. Samples were electrophoresed in 1% agarose gels (see Appendix A1.7.3) in the presence of 2.2 M formaldehyde (BDH, AnalaR, Cat. No. 10113). The gels were then washed with rocking in H_2O for 30 minutes and ethidium-bromide stained mRNA was visualised under ultraviolet (UV) illumination to determine the quality of the mRNA. The gel was blotted onto Hybond N^+ filters (Amersham. Cat. No. RPN 303B) and the mRNA was transferred by capillary action in 10 x SSC (see Appendix A1.7.4) overnight. The nylon membrane was rinsed in 2 x SSC and allowed to air dry prior to cross-linking the mRNA to the filter by exposure to UV light (0.4J/cm^2 in a Hybaid UV crosslinker)

Prior to hybridisation with radiolabelled probes, the filters were incubated for between 2 - 4 hours at 42°C in a plastic bag containing prehybridisation solution (see Appendix A1.7.5). Prehybridisation solution was discarded and replaced with fresh solution. Denatured probes (prepared as described above in section 2.7.3) were added at an activity of 10^6 cpm/ml and hybridisation was allowed to occur for 16 hours at 42°C.

Filters were washed 2x at RT for 10 minutes and then 4x at 55°C with shaking for 30 minutes each in 0.1% SDS, 0.1 x SSC for probes greater than 100 bp. For probes less than 100 bp filters were washed twice for 10 minutes in 2 x SSC, 1% SDS at RT and a further four 10 minute washes at 37°C. Filters were wrapped in plastic and. placed on film for autoradiography with intensifying screens at -70°C for 16 - 24 hours. Hybridisation signals were also detected on phosphor storage screens which were exposed overnight and analysed using a Molecular Dynamics PhosphorImager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Hybridisation signals were quantified using the *GAPDH* signals as a loading control. If nylon membranes were to be reprobed they were stripped by submersion in a solution of boiling 0.1% SDS, 5 mM EDTA, pH 8 and maintained at a temperature of 70 - 80°C for 30 minutes with shaking.

2.8 INTRODUCTION OF c-*kit* cDNA INTO EUKARYOTIC CELLS

2.8.1 (a) cDNAs and expression vectors

Murine c-*kit* cDNA, according to the sequence published by Qui *et al.*, (1988) was generated by Dr. T. Gonda (Hanson Centre for Cancer Research, Adelaide, Australia) from mouse brain cDNA using PCR and cloned between the Xho I - Hind III sites of the retroviral vector pZenneo. pZenneo has previously been described by Johnson *et al.*, (1989) and is diagrammatically represented in Chapter 4, Figure 4.2.1.

Human c-*kit* cDNA, according to the sequence published by Yarden *et al.*, (1987a) harboured between the Asp 718-Not I sites of the pBluescript M13 - (SK) (pBS(SK)) vector was provided by Dr. D. Williams (Immunex Corporation, Seattle, WA).

The expression vector pRSV009/A^+ (Choo *et al.*, 1986) was obtained from Dr J. Wells (Department of Biochemistry, University of Adelaide, Australia). This expression

vector consisted of a neomycin (Neo^R) and dihydrofolate reductase (*DHFR*) genes under the control of SV40 promoters. It consisted of a unique Bam HI restriction site for the cloning of c-*kit* cDNA which lay under the control of the Rous Sarcoma Virus (RSV) LTR (diagrammatically represented in Chapter 5, Figure 5.2.1). A 3 kb Asp 718-Not I c-*kit* fragment excised from pBS(SK) (described above) was blunt ended using Klenow polymerase and ligated into Bam HI digested, end-filled and dephosphorylated pRSVOO9/A^+ (by the methods described in section 2.4) to create pGC1.2.

The retroviral vector pRUFMC1Neo was provided by Drs J. Rayner and T Gonda, (Hanson Centre for Cancer Research, Adelaide, Australia) (Rayner & Gonda, 1994) and contained 5' and 3' LTRs derived from MPSV and the neomycin gene was driven by the f9 polyoma enhancer. The vector is diagrammatically represented in Chapter 5, Figure 5.3.3).

2.8.1 (b) Production of c-*kit* cDNA variants using PCR site-directed mutagenesis

This method was a modification of that published by Hemsley *et al.* (1989). Full length human *c-kit* DNA in the vector pBS(SK) described in section 2.8.1(a) was used as template DNA in a polymerase chain reaction (PCR). The reaction consisted of all the reagents described in section 2.5 except that Pfu DNA polymerase (Stratagene Cat No. 600153) was used instead of AmpliTaq polymerase. The former was used since it possesses a 3'-5' endonuclease activity which prevents mismatch errors from being introduced during PCR amplification. The reaction volume was 100 µl and contained either 40 or 0.5 ng template DNA and 2.5 U Pfu DNA polymerase and the appropriate pair of oligonucleotide primers to produce the deletion of interest. The primers 796 (anti-sense) and 797 (sense) (refer to Table 2.5.2.1 for sequences and locations based on the published sequence by Yarden *et al.*, 1987a) were used to produce a 12 bp deletion between nucleotides 1549 and 1562 in *c-kit* and the primers 1002 (anti-sense) and 1003 (sense) (refer to Table 2.5.2.1) were used to produce a 3 bp deletion between nucleotides 2164 - 2166. These primers immediately flanked the area to be deleted. The PCR cycles were as follows: an initial denaturation cycle of 94°C for 7 minutes and then 25 cycles comprising of 94°C for 1 minute, 45°C for 1 minute and 72°C for 12 minutes and then a final extension cycle of 72°C for 12 minutes.

A 10 µl aliquot of the reaction mix was run on a 0.8% agarose gel to verify whether a 6 kb PCR product (ie c-*kit* + pBS(SK)) was produced. Upon verification 20 µl of the PCR was run on a 1% low melting point Seaplaque agarose gel and the product was gel purified via agarase treatment (see section 2.4.3 (c)). The 5' ends of the purified product were phosphorylated as described in section 2.4.8 prior to self ligation (see section 2.4.9).

The ligation reaction mix was diluted 1:10 in H_2O and 2 µl of the ligation reaction mix was transformed into electrocompetent MC1061 E.*coli* cells (see section 2.4.12). Transformants were initially screened for the presence of a 6 kb plasmid by restriction endonuclease digestion of small scale plasmid preparations (see section 2.4.13). Verification that the deletions had been introduced correctly were performed either by further restriction endonuclease digestion and/or PCR and/or sequencing. The c-*kit* product containing the 12 bp deletion was fully sequenced to verify that it corresponded to the sequence published by Yarden *et al.*, (1987a) (minus the 12 bp). One base change at position 2415 was found in both the template and PCR product where a C was replaced by a T, however this change did not alter the amino acid (Ile) encoded at residue 798. The c-*kit* cDNA generated was designated c-*kit(GNNK-S+)* (GNNK referring to the 4 amino acids encoded by the 12 bp and S referring to the serine amino acid encoded by the 3 bp).

In order to produce c-*kit* cDNA variants c-*kit(GNNK-S-)* and c-*kit(GNNK+S-)* a Dra II-Bgl II fragment which encompassed the 3 bp of interest was excised from c-*kit(GNNK-S+)* (produced as described above) and from the c-*kit* cDNA originally used as a template (now referred to as c-*kit(GNNK+S+)*). This was replaced with a Dra II - Bgl II fragment excised from the PCR product generated by amplification with the primers 1002 and 1003 described above. The latter had been sequenced across the Dra II-Bgl II region to verify that it corresponded to the published sequence (minus the 3 bp). For a diagrammatical representation of the production of the c-*kit* variants refer to Chapter 5, Figure 5.3.1 B

The c-*kit* cDNA variants generated were excised from pBS(SK) using the restriction endonucleases Asp 718 - Not I and end-filled using Klenow DNA polymerase (refer to section 2.4.6). This was cloned into Hpa I digested pRUFMC1Neo vector DNA which had also been dephosphorylated (refer to section 2.4.7). The resulting constructs were designated

pRUF(GNNK+S+), pRUF(GNNK+S-), pRUF(GNNK-S+) and pRUF(GNNK-S-) (diagrammatically represented in Chapter 5, Figure 5.3.3).

2.8.2 Transfection of NIH/3T3 cells via the Calcium Phosphate Technique

This procedure was based on that described by Graham *et al.*, (1980) and used to introduce pGC1.2 into NIH/3T3 cells. Tissue culture dishes (60 mm in diameter) (Falcon, USA, Cat. No. 2002) were seeded with 2 x 10^5 NIH/3T3 fibroblast cells, in a volume of 3 ml DMEM/10% FBS, 24 hours prior to transfection. On the day of transfection dishes were checked for their degree of cell confluency. Cells were no greater than 50 - 60% confluent. 1 hour prior to adding the co-precipitate the medium on the cells was replaced with 3.5 ml of fresh medium. During this time, the co-precipitate was formed (the following volumes are for 1 dish): to a 10 ml conical polystyrene tube (Disposable Products, Australia, 21829) (tube A) were added, 25 µl 2 M $CaCl_2$, 5 - 20 µg circular plasmid DNA which was sterilised by filtering through a spinex filter (Costar, MA, Cat. No. 8160). The volume was made up to 0.5 ml using Tris-EDTA (TE) (1 mM Tris (Sigma, USA, Cat. No. T-1378), 0.1 mM EDTA, pH 7.9). The pH of the TE was checked immediately prior to use, and filter-sterilized through a 0.22 µm filter (Sartorius, Germany, Cat. No. 165 34K). To a 30 ml conical polycarbonate tube (Techno-Plas. South Australia, Australia. Cat. No. C9025 (tube B) were added 495 µl 2 x HEPES Buffered Saline (2 x HBS) (280 mM NaCl, 50 mM HEPES, pH 7.1) and 5µl 150 mM NaH_2PO_4.

The contents of tube A were gently mixed and added dropwise, using a plastic pipette, to tube B while bubbling air through the solution in tube B. The co-precipitate was allowed to stand for 30 minutes at RT. A volume of 1 ml of this solution was added to each dish containing NIH/3T3 cells and incubated in 5% CO_2 at 37°C. The next day the medium/precipitate was removed from each dish, and the cells were subjected to glycerol shock by adding 3 ml/dish of DMEM/10% FBS/15% glycerol (BDH, AnalR, Cat. No. 10116) and incubating at RT for a maximum of 2 minutes. The glycerol was removed and the cells washed twice with TC-PBS. This was then replaced with 3 ml fresh DMEM/10% FBS. The following day the cells in each dish were harvested and distributed into four tissue culture

dishes (10 cm in diameter)(Costar, MA, Cat. No. 3100) in 10 ml DMEM/10% FBS/400 μg/ml Geneticin (G418)(Gibco, USA, Cat. No. 11811-031). Cells were fed twice weekly in the presence of selective medium until the mock transfected cells died. A fraction of the G418 resistant cells were then screened for c-Kit protein expression using either APAAP, immune rosetting or IIF techniques described in section 2.2.

c-Kit expressing clones identified by the immune rosetting method were picked using glass cloning rings. Clones were then placed under methotrexate (Mtx)(Sigma, USA, Cat. No. M-8407) selection forcing the amplification of the dihydrofolate reductase (*DHFR*) and linked c-*kit* genes. DHFR is involved in the catalysis of folate to tetrahydrofolate, a compound involved in nucleotide biosynthesis and glycine synthesis. Mtx is an analog of folate which inhibits DHFR, therefore in order for the cell to survive in the presence of Mtx amplification of *DHFR* occurs along with the linked gene. Untansfected NIH/3T3 cells were sensitive to 100 nM Mtx but cells transfected with pGC1.2 showed resistance to this concentration. pGC1.2 and pRSVOO9/A+ transfected cells were cultured in the presence of 100 nM Mtx for two weeks with at least one harvesting occurring in this time. A fraction of the cells were then placed under 200 nM Mtx selection. Every two weeks a fraction of the resistant cells were cultured in increasing concentrations (2 fold increments) of Mtx generating a series of Mtx resistant cell lines expressing progressively increasing levels of c-Kit protein (described in more detail in Chapter 5).

2.8.3 Transfection via calcium phosphate into ψ2 cells

This protocol is similar to that described above with several modifications. Dishes (6 mm in diameter) were seeded with 2 x 10^5 ψ2 cells 24 hours prior to the transfection such that the cells were 50-60% confluent. On the day of transfection the dishes were fed approximately 2 hours prior to transfecting with 3.5 ml DMEM/10% FBS. During this time the co-precipitate was formed as follows (the following volumes are for 2 dishes): to a 10 ml polystyrene tube were added 62.5 μl 2 M $CaCl_2$, 20 μg plasmid DNA (prepared as described in section 2.4.14 or 2.4.15) and this was made up to a final volume of 500 μl with sterile tissue culture grade Milli-Q-purified H_2O (tube A). To a 20 ml conical polystyrene tube were

added 62.5 µl 2 M NaCl, 7.5 µl 0.1 M NaH_2PO_4, pH 7.0, 50 µl 0.5 M HEPES, pH 7.1 and made up to a final volume of 500 µl with H_2O (tube B). The contents of tube A were added dropwise to tube B whilst bubbling air into tube B. A fine precipitate formed which was left to stand at RT for 30 minutes. A volume of 500 µl of this co-precipitate was added to each dish and incubated at 37°C in 5% CO_2. 24 hours later the cells were glycerol shocked with 1 ml DMEM/15% glycerol for precisely 4 minutes rocking gently every 30 - 60 seconds. This was removed and the cells washed with 5 ml of DMEM and then 4 ml of fresh DMEM/10% FBS were added. The following day the cells were harvested and split 1:20 in DMEM/10% FBS containing 400 µg/ml G418. Cells were fed twice a week and 10 - 12 days after G418 selection, when the mock cells had died, clones and pool populations were harvested.

2.8.4 Retroviral infection of fibroblast cell lines

Supernatant containing defective retrovirus carrying c-*kit* or control virus was collected from ψ2 cells. ψ2 cells were allowed to become confluent and then fresh medium containing no G418 was added to the monolayers. The following day the supernatant was collected and filtered through a 0.45 µm filter (Sartorius, Germany, Cat. No. 165 55K). The filtrate was either used fresh or stored in 1 ml ampoules (Nunc, Denmark, Cat. No. 3-66656) at -70°C.

NIH/3T3, N1 or *Sl/Sl* cells (descibed in section 2.1.3) were seeded into tissue culture dishes (60 mm in diameter) 24 hours prior to infection such that the cells were at 10 - 20% confluency on day of infection. The medium was removed from the dishes and replaced with 1 ml of neat or diluted (1:10 and 1:100) viral supernatant in the presence of 4 µg/ml polybrene. The dishes were incubated for 2 hours at 37°C, rocking every 30 minutes and then 3 - 4 ml fresh DMEM/10% FBS was added. After 48 hours the cells were harvested and neat or diluted (1:10, 1:20 and 1:100) cell suspension was replated into five 60 mm diameter tissue culture dishes in DMEM/10% FBS containing 400 µg/ml of G418. Cells were fed twice weekly until mock infected cells were dead. To determine the titre of the virus colonies were stained with Diff-Quick (Lab Aids, Australia, LP-64851) and counted and the following

formula was applied:

Titre (colony forming units (CFU)/ml) = number of $G418^R$ colonies x dilution of viral supernatant x dilution of cells

The pool of infectants were collected and screened for c-Kit protein expression by IIF and APAAP techniques (see sections 2.2.2 and 2.2.4.).

2.8.5 Retroviral infection of suspension cells

Semi-confluent monolayers of ψ2 cells containing RUF(GNNK+/-S+/-) constructs grown in 25 cm^2 growth area flasks were irradiated at 25 Gray prior to co-cultivation with FDC-P1(SLF) cells or 32D cells (described in section 2.1.3). Tissue culture flasks (25 cm^2 growth area) were seeded with 6 - 10 x 10^5 irradiated ψ2 cells to which were added 3 - 5 x 10^5 FDC-P1(SLF) cells to give a 2:1 ratio of ψ2 to FDC-P1(SLF) cells. To these co-cultures were added various combinations of growth factors (as described in Chapter 6) adjusting the final volume of the co-culture to 4 ml with DMEM/10% FBS. Cells were co-cultivated for 2 - 3 days prior to collecting the non-adherent FDC-P1(SLF) cells from the ψ2 cells and culturing in 5 ml of DMEM/10% FBS/growth factors and 1 mg/ml of G418. Cells were fed twice weekly by centrifuging the cells at 200 g and resuspending in fresh DMEM/10% FBS/growth factors and 1 mg/ml of G418 until the mock infected cells had died. Cells were screened for c-Kit protein expression by APAAP and IIF techniques (see sections 2.2.2 and 2.2.4.)

2.9 TRANSFORMATION ASSAYS

2.9.1 Low serum assay

NIH(mu*kit*) cells and NIH(*neo*) control cells were washed three times in order to remove FBS and then 100 cells/2 ml were aliquoted into the wells of a 6 well plate (Costar, Cambridge, MA Cat. No. 3506) in the presence of 1% or 10% FBS. Cells were also cultured in the presence or absence of 100 ng/ml mSLF in duplicate. Cells were fed twice weekly and after 10 days the colonies were stained with Diff-Quik stain (Lab Aids, Australia, LP-64851) and colonies counted.

2.9.2 Foci assay

epNIH/3T3 c-Kit infectants and control cells were washed and 2 x 10^3 cells were plated into 6 well (35 mm in diameter) plates in 2 ml of medium in the presence or absence of 100 ng/ml mSLF. Cells were fed twice weekly and allowed to become confluent. Cells were stained after 8 - 12 days using the Diff-Quik stain.

2.9.3 Anchorage Independence Assay

Cells were harvested and seeded at 2 x 10^5- 5 x 10^5 cells/75cm^2 growth area tissue culture flasks 24 - 48 hours prior to the assay to achieve approximately 50% confluency on the day of the assay. On the day of the assay cells were harvested in DMEM/10% FBS and centrifuged at 200 g at 25°C for 5 minutes in a Mistral 3000i MSE centrifuge and resuspended at approximately 10^5 cells/ml. Cell concentration and viability were initially determined using a haemocytometer and trypan blue exclusion, however a more accurate determination was later calculated by mixing a 50 µl aliquot of the cell suspensions used in the assay with a known concentration of Standard Brite beads (10^5 beads/ml). The cell concentration was determined by running the suspension through the Profile II flow cytometer and using the following formula:

$$\frac{\text{cell count}}{\text{bead count}} \times \frac{\text{cell sample volume (ml)}}{\text{bead sample volume (ml)}} \times \frac{\text{bead density}}{10^6}$$

The desired number of cells (2 - 10 x 10^3cells/dish and each test was generally performed in quadruplicate) and varying concentrations of recombinant SLF were added to 5 ml (quantity for 5 plates) of ds-IMDM : 0.66% agar (1:1) (prepared as below) in 10 ml polypropylene flat bottomed tubes.

Two densities of agar were prepared, 0.66% and 1.32% w/v, by boiling purified bacto-agar (Difco, USA, Cat. No. 0140-01) in tissue culture grade Milli-Q-purified H_2O for 15 minutes, standing at RT for 15 minutes and then equilibrating in a water bath at 42°C. ds-IMDM (prepared as in section 2.1.1) was prewarmed to 37°C, and to this were added 20% FBS and a 7.5% $NaHCO_3$ solution (prepared in H_2O) at 1/10 the volume of ds-IMDM.

ds-IMDM was mixed 1:1 with 1.32% agar by inverting several times and 2 ml was aliquoted into issue culture dishes (35 mm in diameter)(Corning, NY, Cat. No. 25 000) and allowed to set. This agar layer served as an underlayer to the top layer containing the cells to prevent them from growing on the bottom of the dish. The top layer of semi-solid agar was prepared by mixing ds-IMDM/20% FBS 1:1 with 0.66% agar. The resulting 0.33% semi-solid agar was then added to 10 ml tubes one at a time and placing the bulk of the medium/agar mix at 42°C to prevent it from solidifying during the setting up of the assay. The desired amount of cells and SLF were added to the semi-solid agar via a pipette in a volume contributing less than 10% of the total volume and mixed by pipetting several times with a 5 ml pipette prior to aliquoting. Aliquots of 1 ml of this mixture containing 2 - 10 x 10^3 cells were added on top of the underlayer of semi-solid agar and allowed to set. Dishes were incubated in culture boxes in a humidified atmosphere of 5% CO_2 at 37°C for 2 - 3 weeks. Colonies containing greater than 50 cells were counted using an inverted Olympus microscope. Plating efficiencies (number of colonies/cells plated) were altered according to the number of cells plated as determined by the Standard Brite method. Colonies were fixed with 1 ml of 1.5% v/v gluteraldehyde and stored in an air tight container at 4°C for later perusal.

2.9.4 Injection of *nude* mice

Eight week old female Balb/c *nude* mice were obtained from the Medical School Animal House (University of Adelaide; colonies were initiated from mice bred at the New South Wales University, Australia). Groups of 3 - 4 mice were injected subcutaneously in the hind flank with 10^4, 10^5, 10^6 or 10^7 cells (in 100 µl) using a short 26 gauge needle. Mice were monitored twice weekly for the appearance of solid tumours. The width and length of the tumours were measured weekly. Mice were sacrificed when tumours reached 1 - 2 cm in diameter. Cells were prepared for injection by trypsinisation of 50 - 70% confluent monolayers into DMEM/10% FBS followed by several washes in DMEM/2%FBS. An aliquot of this suspension was made up to 10^5, 10^6, 10^7or 10^8 cells/ml in serum free DMEM.

CHAPTER 3

Responses of the murine myeloid cell line FDC-P1 to soluble and membrane-bound forms of *Steel* factor (SLF)

3.1 INTRODUCTION

The c-*kit* proto-oncogene product and its ligand, *Steel* factor (SLF) are encoded at the mouse *dominant white spotting (W)* and *steel (Sl)* loci respectively (Chabot *et al.*, 1988; Geissler *et al.*, 1988; Huang *et al.*, 1990; Zsebo *et al.*, 1990a). Mutations at these loci give rise to similar phenotypic disorders of haemopoiesis characterised by reduction in stem cell numbers, anaemia and mast cell deficiency (Copeland *et al.*, 1990; Flanagan & Leder, 1990; Russell, 1979; Silvers, 1979; Nocka *et al.*, 1990b). Bone marrow transplantation and *in vitro* co-culture experiments have shown that c-*kit*/*W* mutations affect stem cells intrinsically, whereas *SLF/Sl* mutations affect stromal cells of the haemopoietic microenvironment (McCulloch *et al.*, 1964, 1965; Dexter & Moore, 1977; Russell & Bernstein, 1968).

SLF is produced as both membrane-bound and soluble forms due to alternative mRNA splicing and/or proteolytic cleavage (Anderson *et al.*, 1991; Flanagan *et al.*, 1991; Huang *et al.*, 1992; Toksoz *et al.*, 1992; Pandiella *et al.* 1992; Majumdar *et al.*, 1994). Both forms are produced by bone marrow stromal cells and several fibroblast cell types (Williams *et al.*, 1990; Huang *et al.*, 1990; Nocka *et al.*, 1990a; Anderson *et al.*, 1990; Flanagan & Leder, 1990; Boswell *et al.*, 1990; Jozaki *et al.*, 1991). Deletions at the *Sl* locus have been shown to alter the production of SLF, such that *Sl/Sl*^{d} and *Sl/Sl* mice produce a soluble form only, or no SLF, respectively (Flanagan *et al.*, 1991; Huang *et al.*, 1992). The phenotypes of these mice demonstrate that both forms of SLF are important in the regulation of normal steady-state haemopoiesis and that membrane-bound SLF must provide an essential function which differs quantitatively or qualitatively from that supplied by soluble SLF.

Co-culture of bone marrow derived, IL-3-dependent mast cells on some fibroblast "feeder" layers in the absence of exogenous growth factors has shown that cell-to-cell contact is essential for mast cell support (Fujita *et al.*, 1988, 1989). This cell association has been attributed to the interaction between c-Kit on the mast cells and membrane-bound SLF produced by the "feeder" layers (Flanagan *et al.*, 1991; Adachi *et al.*, 1992). Similar c-Kit/SLF interactions have been demonstrated to be involved in the support of megakaryocytes and B-cells in similar co-culture systems (Avraham *et al.*, 1992; Rolink *et al.*, 1991). Dexter-type long term bone marrow cultures have also indicated the importance

of membrane-bound SLF in the maintenance of normal haemopoiesis. Fibroblast and stromal layers derived from *Sl/Sl^d* mouse embryos, which lack membrane-bound SLF, failed to support normal production of myeloid progenitor cells and mast cells (Dexter & Moore, 1977; Fujita *et al.*, 1989; Boswell *et al.*, 1990), thus paralleling the phenotype of *Sl/Sl^d* mutant mice. Lack of support of progenitor and mast cells on "feeder" layers producing soluble SLF only may simply be a result of insufficient production of diffusable factor i.e. concentrations too low to cause stimulation of these cells. It has been shown that different fibroblast lines can produce soluble SLF in varying amounts and that mast cells can be supported on some fibroblast layers in a contact-independent manner due to their high production of soluble SLF (Jozaki *et al.*, 1991). It appears then that high concentrations of soluble SLF may be able to mimic the action of membrane-bound SLF (Zsebo *et al.*, 1990a).

Soluble recombinant SLF has been shown to synergise with cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), granulocyte colony-stimulating factor (G-CSF) and erythropoietin (Epo) in enhancing colony formation by both human and murine myeloid progenitors, however it has little effect on its own (Broxmeyer *et al.*, 1991a,b; McNiece *et al.*, 1991a; Metcalf & Nicola, 1991). Soluble SLF has also been shown to synergise with IL-3, GM-CSF, interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-9 (IL-9) and gamma-interferon (IFN-γ) to produce enhancement (additive to greater than additive) of the proliferation of a human factor-dependent cell line, M07e (Hendrie *et al.*, 1991).

The murine early myeloid factor-dependent cell line, FDC-P1 (Dexter *et al.*, 1990) expresses c-*kit* (Welham & Schrader, 1991) and is dependent on GM-CSF or IL-3 for growth (Hapel *et al.*, 1984). These properties suggested that co-culture of FDC-P1 cells on fibroblast feeder layers may be supported by SLF in a similar way to mast cells, as described above. Initial experiments performed by Dr. T. Gonda (at the Ludwig Institute for Cancer Research, Melbourne, Victoria, Australia; currently at The Hanson Centre for Cancer Research, Adelaide, Australia) demonstrated that FDC-P1 cells were supported by +/+ fibroblasts but not *Sl/Sl* or *Sl/Sl^d* fibroblasts in a contact-dependent and GM-CSF-independent manner. Thus, support of FDC-P1 cells was attributed to the interaction of c-Kit with membrane-bound SLF

Table 3.2.1: Co-culture of FDC-P1 cells on +/+ and *Sl/Sl* mutant fibroblast cell lines

Cell line	Genotype	Antiserum[1]	Cell No. (x 10^4 /ml) Day4	Day7
$WCB6F_1$-3T3-1	+/+	-	46	30*
$WCB6F_1$-3T3-1	+/+	+	3.5	53
$WCB6F_1$-3T3-2	+/+	-	27	84
$WCB6F_1$-3T3-2	+/+	+	<0.5	7
$WCB6F_1$-3T3-5	*Sl/Sl^d*	-	ND	<0.5
$WCB6F_1$-3T3-5	*Sl/Sl^d*	+	ND	<0.5
$WCB6F_1$-3T3-6	*Sl/Sl^d*	-	ND	20*
$WCB6F_1$-3T3-6	*Sl/Sl^d*	+	ND	<0.5
WCB6F1-3T3-7	*Sl/Sl^d*	-	ND	1
WCB6F1-3T3-7	*Sl/Sl^d*	+	ND	<0.5
VSOP	*Sl/Sl*	-	ND	13
VSOP	*Sl/Sl*	+	ND	<0.5

Assay performed as described in materials & methods section 2.3.2. Data represents results obtained from a typical experiment.

FDC-P1 cells were also assayed with WEHI-3 conditioned medium alone, as a positive control for cell growth. No cell growth was observed in the absence of factor.

[1]antisera used were: + = anti-GM-CSF serum, - = anti-casein serum (negative control)

ND = not determined due to low cell numbers

* lower cell numbers due to cell overgrowth

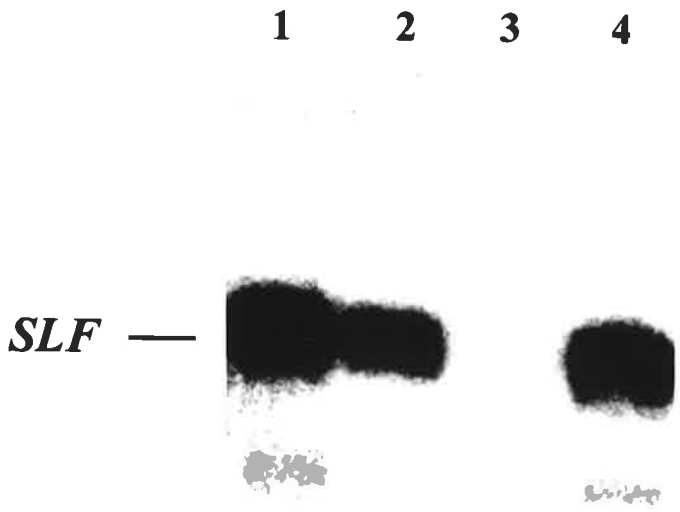
produced by the +/+ cells; soluble SLF produced by the latter or *Sl/Sl^d* fibroblasts was unable to support proliferation. These results formed the basis for further analysis of the responsiveness of FDC-P1 cells to soluble recombinant murine SLF. As FDC-P1 cells were dependent on GM-CSF and IL-3, they provided a useful model for also studying the interactions between the c-Kit receptor pathway and these haemopoietic growth factors.

RESULTS

3.2 *Summary of initial studies demonstrating GM-CSF-independent but contact-dependent growth of FDC-P1 cells on +/+ fibroblasts.*

Contact-dependent support of mast cells when co-cultured with fibroblast feeder layers had been demonstrated to be dependent on the interaction between c-Kit/membrane-bound SLF (Flanagan *et al.*, 1991; Adachi *et al.*, 1992). Initial experiments carried out by Dr. T. Gonda suggested that similar interactions were necessary for the support of the c-Kit expressing, factor-dependent cell line FDC-P1. These conclusions were drawn from initial experiments involving the co-culture of FDC-P1 cells with a panel of fibroblastoid cell lines derived from WCB6F$_1$ +/+ and *Steel* mutant embryos (WCB6F$_1$: *Sl/Sl^d*, and *Sl/Sl*) (Fujita *et al.*, 1989). Because fibroblasts frequently produce significant amounts of GM-CSF, which also allows FDC-P1 cells to proliferate, these experiments were carried out in both the presence and absence of neutralising rabbit antiserum to GM-CSF. (Fibroblasts are not believed to produce significant amounts of IL-3, as confirmed below). On days 4 and 7 after initiating the co-cultures, viable FDC-P1 cells were counted. As shown in Table 3.2.1, in the absence of anti-GM-CSF most of the fibroblast layers tested (WCB6F$_1$ +/+ 3T3-1,-2, WCB6F$_1$ *Sl/Sl^d* 3T3-6 and *Sl/Sl*) were able to stimulate proliferation of FDC-P1 cells to varying degrees. However, in the presence of neutralising levels of anti-GM-CSF, only +/+ fibroblasts (which express SLF mRNA as shown by Northern blotting; see Figure. 3.2.1) were able to support FDC-P1 cell growth. This indicated that the factor responsible for maintaining FDC-P1 cells in the absence of GM-CSF was not produced by *Sl/Sl^d* and *Sl/Sl* fibroblasts, strongly suggesting that the factor was SLF. Another early myeloid IL-3-dependent cell line, 32D, which lacks c-*kit* expression (Nocka *et al.*, 1990), was not

Figure 3.2.1: Expression of *SLF* mRNA in murine fibroblast cell lines. 2 μg of poly A+ selected mRNA was prepared from NIH/3T3 (lane 1), +/+ (lane 2), *Sl/Sl* (lane 3) and *Sl/Sl*^{d} (lane 4) cell lines and was analysed by agarose gel electrophoresis and blot hybridisation (as described in section 2.7.5). Hybridisation was performed using a full length murine *SLF* probe (refer to section 2.7.2).


1
2
3
4
SLF

supported by +/+ or *Sl* mutant cell lines in co-culture, but was able to proliferate in IL-3-containing WEHI-3 conditioned medium, verifying the lack of IL-3 production by these fibroblasts (data not shown).

Of particular interest was the observation that $WCB6F_1$+/+ cells but not $WCB6F_1$*Sl/Sl*^{d} cells could support GM-CSF-independent FDC-P1 growth. Northern blot analysis (Figure 3.2.1) demonstrated that both +/+ and *Sl/Sl*^{d} fibroblasts express *SLF* mRNA, whereas *Sl/Sl* cells totally lack *SLF* transcript, as expected (NIH/3T3 cells are another embryonic fibroblast line from +/+ mice which also expresses *SLF* mRNA). Thus, the major difference between $WCB6F_1$+/+ and *Sl/Sl*^{d} fibroblasts appears to be the production of the membrane-bound form of SLF.

To further investigate the role of membrane-bound versus soluble SLF Dr. T. Gonda examined whether GM-CSF-independent support of FDC-P1 cells by +/+ fibroblasts was contact-dependent by separating the two cell types with an agar interlayer. Separation of the FDC-P1 cells from the fibroblasts slightly reduced the proliferation of FDC-P1 cells (by 32% on day 5 and 18% on day 8 of culture) compared to cultures in the absence of the agar (Table 3.2.2). Cell growth in the presence of agar could be attributed to GM-CSF diffusing through the agar layer. In the absence of GM-CSF, however, it appeared that the activity of +/+ fibroblasts was contact-dependent, since in the presence of anti-GM-CSF and an agar interlayer FDC-P1 cell proliferation was reduced by 91-92%. To further verify that GM-CSF-independent growth of FDC-P1 cells was contact-dependent, conditioned medium collected from +/+ and *Sl/Sl*^{d} fibroblasts was unable to support the growth of FDC-P1 cells in the presence of GM-CSF antiserum but was active in the absence of antibody (data not shown).

3.3 *FDC-P1 responsiveness to soluble SLF*

The initial results described above (in Tables 3.2.1 and 3.2.2) suggested that anti-GM-CSF resistant FDC-P1 cell growth, obtained only with +/+ fibroblasts, was contact-dependent and was most likely due to membrane-bound SLF, known to be produced by these cells but not by *Sl/Sl*^{d} or *Sl/Sl* fibroblasts. It seemed possible that FDC-P1 cells were

Table 3.2.2: Contact-dependence of GM-CSF independent FDC-P1 cells on +/+ fibroblasts

		Cell No. (x 10^4/ml)	
Agar	Antiserum[1]	Day 5	Day 8
+	+	1	2
+	-	34.5	64
-	+	16	32
-	-	51	78

Assay performed as described in the materials & methods section 2.3.2. Data represents results obtained from a typical experiment.

[1]antisera used were:

+= anti-GM-CSF serum

-= anti-casein serum (negative control)

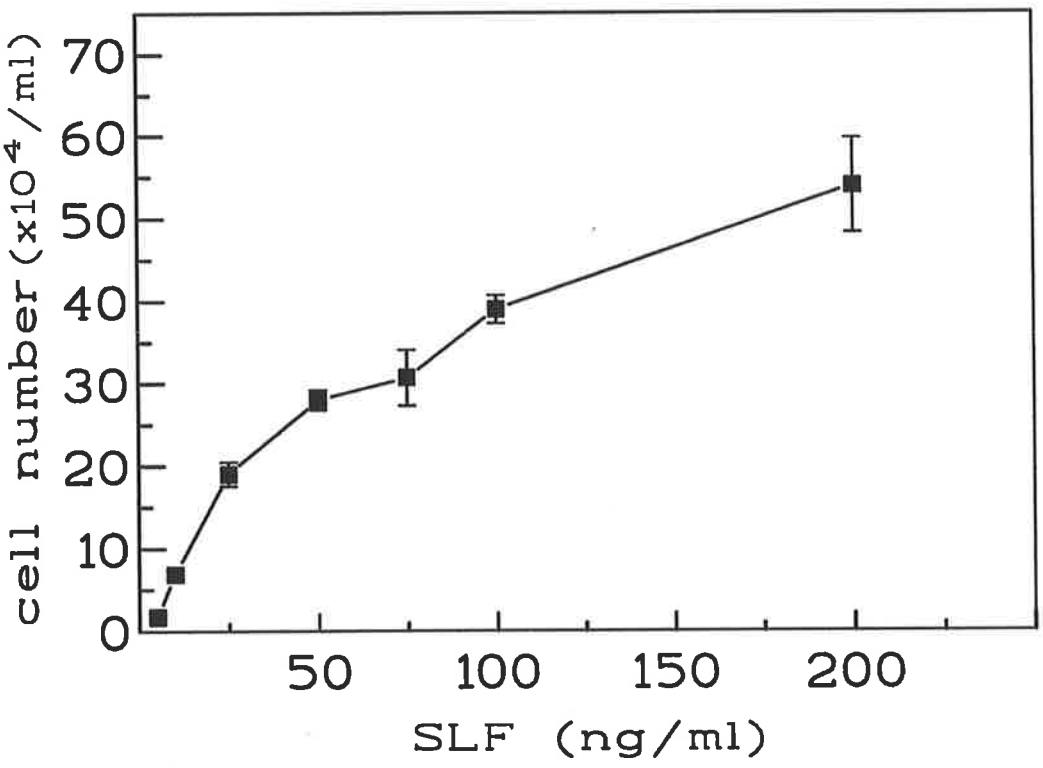
also capable of responding to soluble SLF alone, but that the levels produced by +/+ and *Sl/Sl*^{d} fibroblasts were simply insufficient to stimulate FDC-P1 growth. The cloning of SLF in 1990 (Anderson *et al.*, 1990; Huang *et al.*, 1990; Nocka *et al.*, 1990; Zsebo *et al.*, 1990) and the subsequent availability of soluble recombinant murine SLF enabled us to further this investigation by assaying the responsiveness of FDC-P1 cells to high concentrations of soluble SLF. FDC-P1 cells growing in GM-CSF (80 U/ml) in liquid culture (hereafter termed "FDC-P1(GM)") were washed to remove residual factor and assayed for proliferation in response to increasing concentrations of SLF alone. SLF levels of 100 ng/ml were not effective in supporting FDC-P1 proliferation, with only a few viable cells remaining on day 4 (data not shown).

Similarly attempts to establish cultures of FDC-P1(GM) cells in SLF alone after washing the cells to remove residual GM-CSF, were not successful. However, by "weaning" the cells off GM-CSF slowly over a period of 3 weeks in the presence of 40-100 ng/ml SLF, cultures capable of proliferating in SLF alone (FDC-P1(SLF)) were obtained (Figure. 3.3.1). This required relatively high concentrations of SLF (50-100 ng/ml) compared to the levels of GM-CSF (40 U/ml≈ 200 pg/ml) required to achieve maximal proliferation (refer to Figure. 3.4.1A.). These cells were stably maintained in culture in SLF alone for 6 months and were used in further experiments described in this Chapter. Histological staining of these cells showed no distinguishable changes in morphology compared to FDC-P1 cells grown in culture in GM-CSF or IL-3.

3.4 *SLF increases the proliferation of FDC-P1 cells in synergy with GM-CSF or IL-3*

It is known that SLF potently synergises with a number of growth factors on normal human and murine haemopoietic progenitor cells and on the human factor-dependent cell line, M07e (Broxmeyer *et al.*, 1991; Broxmeyer *et al.*, 1991; McNiece *et al.*, 1991); Metcalf & Nicola, 1991; Hendrie *et al.*, 1991). Therefore we examined whether SLF could stimulate enhanced proliferation of FDC-P1 cells in combination with GM-CSF or IL-3. FDC-P1(GM) cells were washed to remove residual factor and assayed in increasing concentrations of GM-CSF in the presence or absence of 40 ng/ml of SLF for 4 days, at which time viable cells

Figure 3.3.1: Dose-response of FDC-P1(SLF) cells to incremental concentrations of recombinant murine SLF. Cells were washed three times to remove residual SLF and 10^4 cells plated in 500 µl medium containing varying concentrations of SLF as indicated. Cultures were incubated for 4 days in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were counted in a haemocytometer and viability determined by trypan blue exclusion. Data represented as the mean ± SEM of duplicate replicates.


cell number (x10⁴/ml)
70
60
50
40
30
20
10
0
50
100
150
200
SLF (ng/ml)

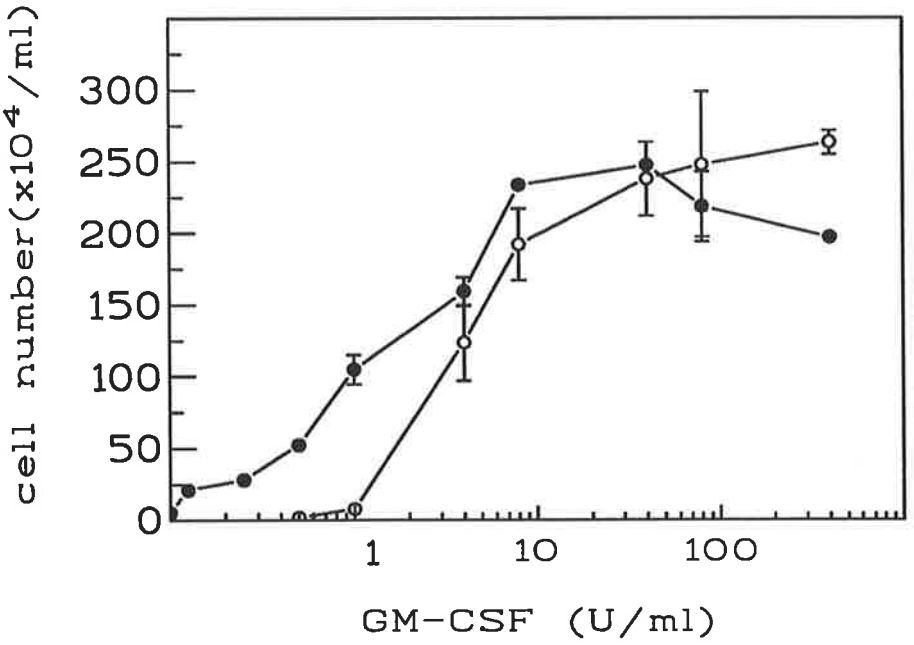
were counted. At low levels of GM-CSF strong synergy with SLF was observed (Fig. 3.4.1A), with proliferation up to 32-fold higher at 0.5 U/ml GM-CSF; the effect of SLF was lost above approximately 40 U/ml GM-CSF. Similar, though less pronounced synergy (2 to 10-fold) of SLF with suboptimal levels of IL-3 was seen on FDC-P1 cells propagated in IL-3 (Fig. 3.4.1 B). Synergy between SLF and GM-CSF was also demonstrated in assays of colony formation by FDC-P1 cells plated in semi-solid medium in the presence of increasing concentrations of GM-CSF with or without 40 ng/ml SLF (performed by Liz MacMillian, The Hanson Centre for Cancer Research, Adelaide, Australia; data not shown). At low levels of GM-CSF (0.3 U/ml) a 2.6 fold increase in colony number was observed and again the effect of SLF on colony numbers diminished with increasing concentrations of GM-CSF. Colonies which grew in the presence of GM-CSF and SLF also appeared larger than those grown in GM-CSF alone (data not shown).

3.5 *Downregulation of c-Kit by GM-CSF and IL-3*

The data described above demonstrating that FDC-P1(SLF) cells were more responsive to SLF than FDC-P1(GM) led us to assay the effects of SLF, IL-3 and GM-CSF alone on the three FDC-P1 cell populations, i.e. FDC-P1 cells which had been maintained in SLF, GM-CSF or IL-3. FDC-P1(SLF) cells were more responsive to SLF than FDC-P1(GM) and also FDC-P1(IL-3) cells (Figure. 3.5.1A). At a SLF concentration of 100 ng/ml, FDC-P1(SLF) exhibited 9-10 fold greater ^{3}H-thymidine incorporation than FDC-P1(GM) cells and 5 fold greater incorporation than FDC-P1(IL-3) over background levels (Figure. 3.5.1A). FDC-P1(GM) and FDC-P1(IL-3) generally demonstrated only basal levels of ^{3}H-thymidine incorporation (compared to in the absence of factor) at all concentrations of SLF (except FDC-P1(IL-3) cells in 100 ng/ml SLF). In contrast, all three populations responded well to GM-CSF and IL-3 (Figure. 3.5.1B, C). At saturating levels of GM-CSF all cell populations proliferated equally well, while at low levels of GM-CSF (0.25 - 1 U/ml) FDC-P1(IL-3) cells incorporated slightly more ^{3}H-thymidine than FDC-P1(SLF) or FDC-P1(GM) cells. Exposing the three cell populations to IL-3 resulted in greater proliferation by FDC-P1(IL-3) compared to FDC-P1(GM), and in turn, both of these

Figure 3.4.1: Influence of SLF on FDC-P1 cell proliferation in combination with GM-CSF or IL-3. FDC-P1 cells were washed three times to remove residual factor in which the cells had been maintained. Cells were plated at 10^4 cells/well in 500 μl of medium containing varying concentrations of (A) GM-CSF or (B) IL-3 in the presence of 40 ng/ml SLF (•) or the absence of SLF (o). Data represented as the mean ± SEM of duplicate replicates.

A


cell number(x10⁴/ml)
300
250
200
150
100
50
0
1
10
100
GM-CSF (U/ml)

B

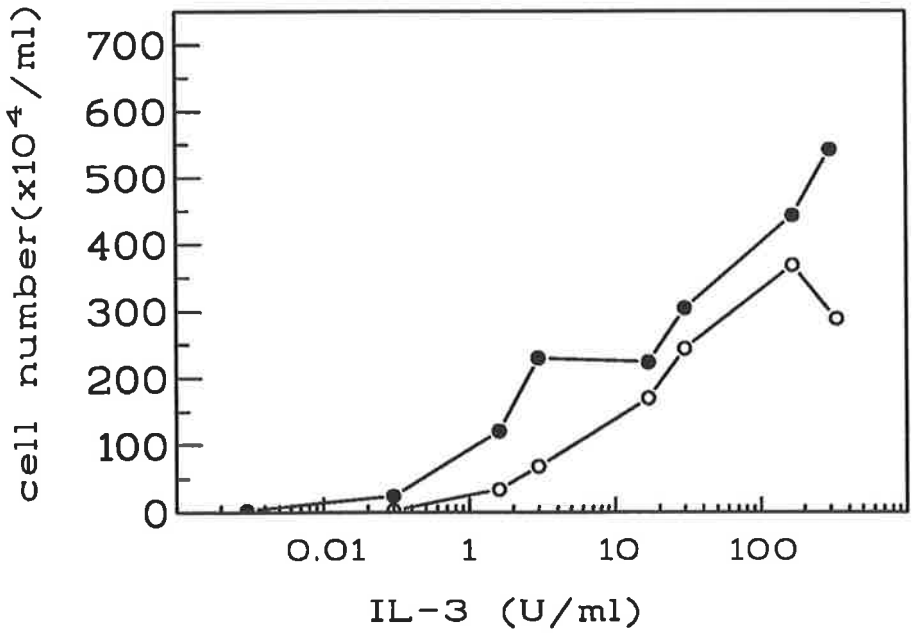
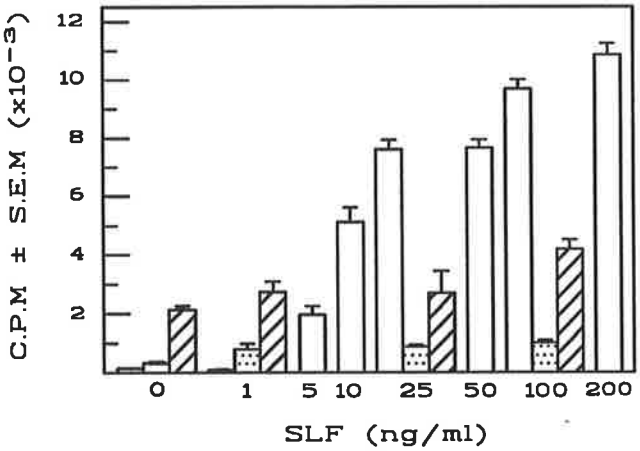
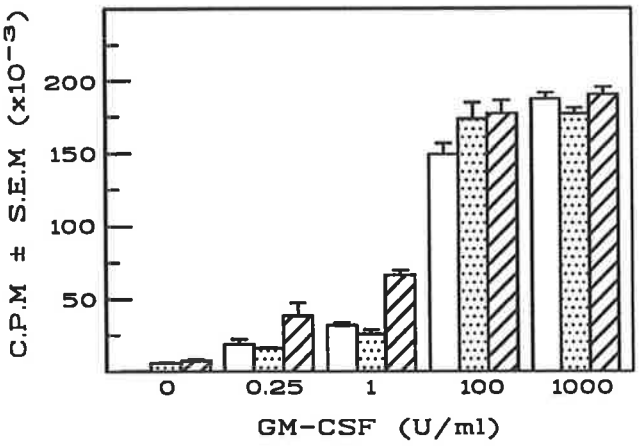

cell number(x10⁴/ml)
700
600
500
400
300
200
100
0
0.01
1
10
100
IL-3 (U/ml)

Figure 3.5.1: Influence of SLF, GM-CSF and IL-3 alone on each of 3 FDC-P1 cell populations. FDC-P1 cells were washed three times to remove residual factor in which the cells had been maintained. FDC-P1(SLF) (white bar), FDC-P1(GM) (dotted bar), and FDC-P1(IL-3) (hatched bar) cells were plated in quadruplicate at 10^4 cells/well into 96 well plates in the presence of increasing concentrations of SLF (**A**), GM-CSF (**B**) or IL-3 (**C**). Cultures were incubated for 48 hours at 37°C in 5% CO_2. Proliferation was measured by incorporation of ^{3}H-thymidine over the last 18 hours of culture. Data represented as the mean count per minute (cpm) ± SEM.

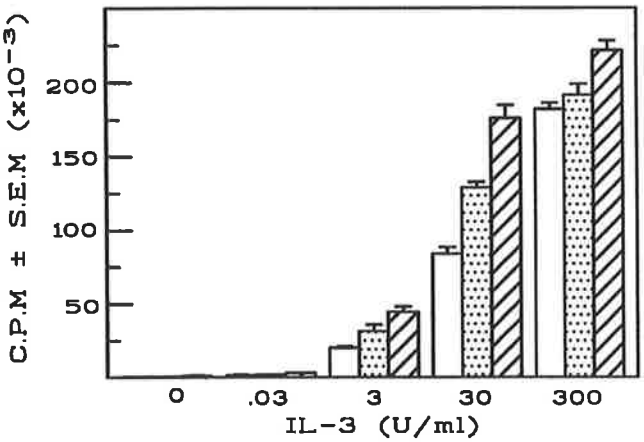
A


C.P.M ± S.E.M (x10⁻³)
12
10
8
6
4
2
0
1
5
10
25
50
100
200
SLF (ng/ml)

B


C.P.M ± S.E.M (x10⁻³)
200
150
100
50
0
0.25
1
100
1000
GM-CSF (U/ml)

C


C.P.M ± S.E.M (x10⁻³)
200
150
100
50
0
.03
3
30
300
IL-3 (U/ml)

populations showed a greater response than FDC-P1(SLF) at intermediate but not at high levels of IL-3 (Figure. 3.5.1C). Thus, growth of the cells in SLF did not down-regulate responsiveness to GM-CSF and only minimally affected responsiveness to IL-3, i.e. a distinct preferential response to the factor the cells had been maintained in was not a general phenomenon.

Analysis of c-*kit* mRNA in FDC-P1(SLF), FDC-P1(GM) and FDC-P1(IL-3) cells revealed the expected 5.5 kb mRNA (Qui *et al.*, 1988). Quantitation of mRNA levels indicated an approximate 7-fold and 2.5-fold decrease in c-*kit* transcripts in FDC-P1(GM) and (IL-3) cells respectively, compared to FDC-P1(SLF) (Figure. 3.5.2). The level of surface c-Kit protein on these cells was determined using a polyclonal rabbit antiserum to murine c-Kit, detected with an α-rabbit IgG conjugated to FITC and subsequent flow cytometry analysis. However, little difference was seen between c-Kit protein levels on the three populations determined by comparing mean fluorescence intensities (Table 3.5.1). This appeared to reflect down-regulation in the case of FDC-P1(GM) and FDC-P1(IL-3) because transfer of these cells to a low concentration of GM-CSF (1 U/ml) or IL-3 (3 U/ml) respectively (over a 24 hour period), increased the level of cell surface c-Kit protein approximately two-fold. Similarly, increased expression of c-Kit protein was seen on FDC-P1(SLF) cells transferred to 1 U/ml GM-CSF (Table 3.5.1). We suspect that the low level of cell surface c-Kit protein on FDC-P1(SLF) cells is due to ligand-induced receptor internalisation in the culture containing this factor or possible interference of the c-Kit antiserum binding to SLF-occupied receptors. On the other hand, the low levels on the cell populations cultured in "normal" levels of GM-CSF or IL-3 reflect the lower mRNA levels.

3.6 DISCUSSION

Murine factor-dependent FDC-P1 cells proliferate in response to GM-CSF, IL-3, or as shown in the initial studies described in this chapter, when co-cultured with certain fibroblastoid cell lines. With some feeder cell lines, proliferation of FDC-P1 cells occurred in the presence of blocking antibodies to GM-CSF, indicating that another cell factor was involved. That this factor was the c-Kit ligand, SLF, was indicated by the failure of *Sl/Sl* or

Figure 3.5.2: Expression of c-*kit* mRNA in FDC-P1 cells. 2μg of poly A+ mRNA was prepared from FDC-P1 cells which had been grown in SLF (lanes 1 and 5), GM-CSF (lanes 2 and 4) or IL-3 (lane 3) in culture. mRNA was visualised by agarose gel electrophoresis and northern blot hybridisation (refer to section 2.7.5). Hybridisation was performed using a full length murine c-*kit* probe and a 780 bp human *GAPDH* fragment labelled with ^{32}P. Hybridisation signals were recorded and quantified by PhosphorImager analysis using *GAPDH* signals to determine comparative loading. Lanes 1-3: Experiment 1; Lanes 4 and 5: Experiment 2 showing more equal loading of mRNA from FDC-P1(GM) and FDC-P1(SLF) cells.

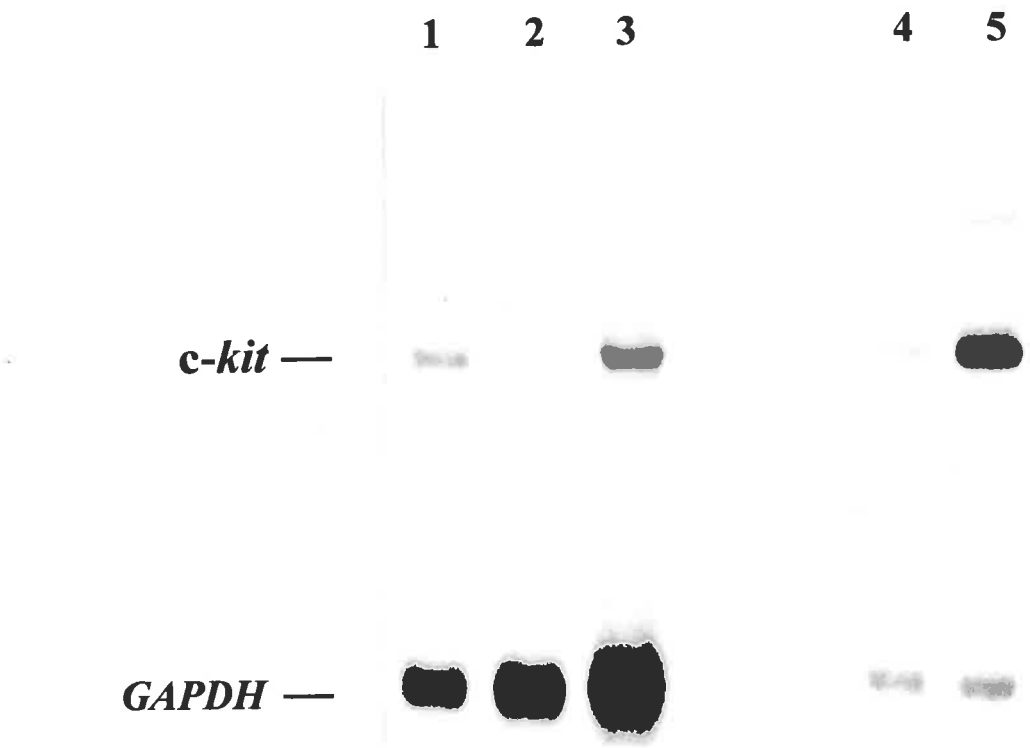

1
2
3
4
5
c-*kit* —
GAPDH —

Table 3.5.1: Effects of GM-CSF, IL-3 and SLF on c-Kit protein expression on FDC-P1 cells.

Cells	Factor[1]	Mean Fluorescence intensity	
		n.r.s.[2]	α-c-Kit
FDC-P1(GM)	GM-CSF (80 U/ml)	2.0	5.2
FDC-P1(IL-3)	IL-3 (300 U/ml)	1.7	3.7
FDC-P1(SLF)	SLF (50 ng/ml)	2.1	5.1
FDC-P1(GM)	GM-CSF (1 U/ml)	1.9	7.7
FDC-P1(IL-3)	IL-3 (3 U/ml)	1.7	5.8
FDC-P1(SLF)	GM-CSF (1 U/ml)	1.5	7.8

[1]FDC-P1 cells grown in either the usual maintenance levels of GM-CSF, IL-3 or SLF or reduced levels of factor, as indicated for 24 hours, prior to assay.

[2]Pre-immune normal rabbit serum (n.r.s.) used as negative control

Sl/Sl^{d} fibroblasts to support FDC-P1 proliferation in the presence of anti-GM-CSF. Nevertheless, FDC-P1 cells maintained in GM-CSF proliferated poorly in SLF alone in a 3-day assay, and in fact the viability of the cultures progressively declined over this period. This could indicate that FDC-P1 cells require membrane-bound as distinct to soluble SLF; that SLF could only act in synergy with residual low levels of GM-CSF (i.e. GM-CSF that escapes neutralisation by the antibody); or that other fibroblast-derived factors are involved.

Consistent with a role for membrane-bound SLF, WCB6F$_1$ *Sl/Sl*^{d} fibroblasts, which produce only soluble SLF (Flanagan *et al.*, 1991; Huang *et al.*, 1992), did not support FDC-P1 proliferation although they were shown to produce abundant *SLF* mRNA and thus, we assume, protein. Furthermore, separation from the WCB6F$_1$+/+ fibroblast feeder cells by an agar interlayer in the presence of anti-GM-CSF prevented FDC-P1 growth. The importance of membrane-bound SLF observed in these experiments parallels *in vivo* observations in *Sl/Sl*^{d} mice which have profoundly impaired defects in haemopoiesis. Similar observations to those reported here have previously been made on cultures of IL-3-dependent murine primary mast cells on +/+ and *Sl/Sl*^{d} fibroblast feeder layers (Fujita *et al.*, 1988, 1989). In contrast to our results, Kittler *et al.*, (1992) reported that the growth of FDC-P1 cells on irradiated Balb/c bone-marrow stromal cells was completely blocked by anti-GM-CSF despite the fact that these cells contained *SLF* mRNA. We find it difficult to reconcile these results with ours but the difference may be due to the production of predominantly soluble SLF, arising from proteolysis (Flanagan *et al.*, 1991; Huang *et al.*, 1992; Pandiella *et al.*, 1992) rather than membrane-associated SLF by the stromal cells used by Kittler *et al.* (1992).

Culture of FDC-P1 cells with combinations of SLF and GM-CSF, as well as SLF and IL-3, showed strong synergy in supporting cell proliferation, especially at low levels of GM-CSF or IL-3 which were relatively ineffective alone. It is possible that, as well as initiating direct signalling pathways, membrane-bound SLF may have acted by mediating intimate contact between FDC-P1 cells and fibroblasts (as also seen with mast cells (Adachi *et al.*, 1992)) and thereby facilitating the action of residual amounts of GM-CSF not neutralised by the anti-GM-CSF. Direct contact could also enable the delivery and synergistic action of small amounts of other, as yet unidentified cytokines produced by fibroblasts.

"Anchor factors" such as SLF have dual roles acting as growth factors as well as adhesion molecules (Gordon, 1991; Lowry *et al.*, 1992). It has been suggested that membrane-associated SLF may "anchor" haemopoietic stem cells to niches within the stromal microenvironment. Thus, enabling them to respond to subliminal concentrations of other cytokines that can act in synergy with SLF, resulting in proliferation and differentiation (Lowry *et al.*, 1992). Alternatively, it has recently been demonstrated that membrane-bound SLF induces a more persistent tyrosine kinase activation of the c-Kit receptor, expressed by M07e cells, in comparison to soluble SLF (Miyazawa *et al.*, 1995). The prolonged activation of the c-Kit receptor by membrane-bound SLF is presumably due to the inability of the receptor/membrane-bound SLF complex to be internalised and degraded. This is consistent with results described by Toksoz *et al.* (1992) which described that soluble SLF produced only transient support of haemopoiesis in long term cultures compared to the longer support achieved by membrane-bound SLF. The more persistent activation of c-Kit achieved by membrane-bound SLF and/or the ability of SLF to synergise with low levels of GM-CSF are both consistent with the results described in this Chapter.

Although soluble SLF produced by the "feeder" layers appeared to be ineffective in maintaining FDC-P1 cells, Zsebo *et al.* (1990a) demonstrated that soluble recombinant SLF, if provided at high enough concentrations, could partially compensate for the loss of membrane-bound SLF in *Sl/Sl*^{d} mice in reducing their macrocytic anaemia, and promoting mast cell accumulation at the site of injection. To further investigate the ability of soluble SLF to maintain FDC-P1 cells, cultures grown in GM-CSF were progressively weaned from GM-CSF to SLF over a period of 3 weeks. In this way cultures stably maintained by SLF alone were obtained. In contrast to cells maintained in GM-CSF or IL-3, these cells displayed strong proliferation in response to SLF, as well as GM-CSF or IL-3, in ^{3}H-thymidine incorporation assays. The response of the three cell populations to GM-CSF and IL-3 were similar except that IL-3-cultured cells showed better growth than GM-CSF -or SLF-cultured cells at sub-optimal levels of either cytokine.

The poor biological response of GM-CSF and, to a lesser extent, IL-3-cultured cells to SLF compared with SLF-cultured cells can probably be explained by reduced levels of c-*kit*

mRNA and protein. Downregulation of c-*kit* mRNA in FDC-P1 cells by GM-CSF and IL-3 has been reported by Welham and Schrader (1991) who demonstrated that reducing the levels of GM-CSF or IL-3 over a 20 hour period resulted in an increase in c-*kit* mRNA and protein. In the experiments described in this Chapter, the levels of c-*kit* mRNA in FDC-P1 cells maintained in GM-CSF and IL-3 were, respectively, ~7-fold and 2.5-fold lower than in cultures maintained with SLF alone. It could be suggested that the poor growth in SLF of FDC-P1 cells which have been maintained in GM-CSF or IL-3 is a consequence of this downregulation, and conversely that increased levels of c-*kit* mRNA (due to initial selection in low concentrations of GM-CSF) may have allowed selection of FDC-P1 cells which proliferate in SLF alone. Surprisingly, there was little difference between c-Kit protein levels as measured on intact cells by flow cytometry, although this may have been due to ligand-induced receptor internalisation by the FDC-P1(SLF) cells or interference of antibody binding to ligand occupied receptors. However, the fact that FDC-P1(SLF) and FDC-P1(GM) cells showed similar levels of surface expression when transferred to low concentrations of GM-CSF indicates that selection for growth in SLF did not select for "permanent" enhanced expression of c-Kit. Moreover, it is interesting to note that c-Kit expression by FDC-P1(GM) cells was sufficient to promote strong synergy of SLF with GM-CSF or IL-3 even though these cells failed to proliferate in SLF alone. The gradual removal of GM-CSF to produce cultures able to proliferate in SLF alone (FDC-P1(SLF)) may have been aided by this strong synergy of SLF with low levels of GM-CSF as well as the upregulation of c-*kit*. These observations raise the possibility that the two populations, FDC-P1(SLF) and FDC-P1(GM), may differ at a point in the signalling pathway downstream of the receptor. Alternatively, the synergistic action of SLF may require a lower "strength of signal" than the complete proliferative response.

In another report, selection of a colony-stimulating factor-1 (CSF-1) responsive subline of FDC-P1 was shown to be accompanied by marked upregulation at the mRNA and protein level of the CSF-1 receptor (encoded by c-*fms*) (Gliniak & Rohrschneider,.1990), a member of the receptor tyrosine kinase family with strong homology to c-*kit* (Qui *et al.*, 1988). As in our experiments, these cells retained the ability to grow in GM-CSF and IL-3, in

which case CSF-1 receptor expression was suppressed at the mRNA level. However, CSF-1 responsive FDC-P1 cells showed characteristics of monocytic differentiation, whereas FDC-P1(SLF) cells were morphologically indistinguishable from FDC-P1 cells maintained in GM-CSF or IL-3.

Although the effect of SLF on GM-CSF and IL-3 receptor levels has not been directly determined, it seems unlikely that they are markedly affected since FDC-P1 cells maintained in SLF proliferated well in response to GM-CSF and IL-3. In another report it was shown that SLF had no effect on the expression of a panel of cytokine receptors including GM-CSF and IL-3, expressed on purified progenitor cells (Testa *et al.*, 1993). Thus the proliferation data described in this Chapter indicate that the regulatory interactions between SLF and these cytokines are essentially unidirectional. Hierarchical downregulation of CSF receptors at the level of ligand binding was demonstrated by Walker *et al.* (1985) although the mechanism by which this occurred could not be determined at that time. The studies described here indicate functional downregulation of c-k*it* in FDC-P1 cells and are consistent with this effect being mediated at the RNA level.

To conclude, the studies described in this Chapter have established that FDC-P1 cells respond to SLF in a number of ways that closely resemble the response of normal haemopoietic progenitor cells in that 1) they can respond differentially to membrane-bound and soluble SLF produced by fibroblast "feeder" cells and 2) they can show synergistic responses to combinations of SLF and GM-CSF or IL-3. Thus this system, constitutes a simplified model of haemopoietic cell/stromal cell interactions. The studies have also demonstrated that upregulation of c-*kit* expression in the absence of GM-CSF has been shown to be functionally significant converting the SLF unresponsive FDC-P1 cells to SLF responsive. The effect of an increase in c-*kit* expression will be further addressed throughout the remainder of this thesis.

CHAPTER 4

The transforming potential of the murine c-*kit* proto-oncogene.

4.1: INTRODUCTION

As demonstrated by the *W* and *Sl* mutant mice, the c-Kit/SLF interaction is essential for normal haemopoiesis. The c-*kit* proto-oncogene product is expressed on very early progenitor cells within the bone marrow. During haemopoiesis the c-Kit receptor is lost as progenitor cells differentiate into more mature lineage-committed cells, except for mast cells which retain very high levels of c-Kit expression (reviewed in Galli *et* al., 1994). Due to a disruption in haemopoiesis, patients with acute myeloid leukaemia (AML) accumulate within their bone marrow blast cells which have been blocked early in the differentiation process at a stage still retaining c-Kit expression. Ashman *et al.,* (1988) demonstrated that within a sub-group of adult AML patients at presentation, 25% displayed high levels of surface c-Kit protein on their blast cells, as detected by the anti-c-Kit monoclonal antibody (mAb), YB5.B8. High levels of c-Kit expression on the blast cells of these patients correlated with their resistance to chemotherapy regimens and as a result had a poorer prognosis.

Similarly, overexpression of other growth factor receptors belonging to the tyrosine kinase family have been reported to be associated with human malignancies. In particular, the HER-2/*neu* receptor has been shown to be overexpressed in breast and ovarian cancers (Slamon *et al.*, 1987, 1989). Overexpression of the *HER-2/neu* (or *c-erbB2*) gene in these patients is mainly due to gene amplification and does not involve mutations within the coding region. The overexpression of the normal *HER-2/neu* gene serves as a prognostic marker and these patients also respond poorly to chemotherapy. The potential role of the overexpression of the HER-2/*neu* and the EGF receptors in carcinogenesis was demonstrated by their ability to induce transformation of NIH/3T3 cells. Cells expressing low receptor levels did not display transformed behaviour, in contrast to those expressing high levels, which were also tumourigenic in *nude* mice (Hudziak *et al.*, 1987; Velu *et al.*, 1987; Di Fiore *et al.*, 1987b). In the case of the EGFR transformation was ligand-dependent. However that induced by HER-2/*neu* receptor overexpression may be due to ligand-independent dimerization (Brandt-Rauf *et al.*, 1990; Samanta *et al.*, 1994), although there is the possibility that NIH/3T3 cells may produce a ligand to the HER-2/*neu* receptor (Lee *et al.*, 1989).

Prior to the isolation of SLF, the ligand to the c-Kit receptor, Lev *et al.*, (1990) demonstrated that a chimeric receptor consisting of the extracellular domain of the human EGFR and the transmembrane and cytoplasmic domains of the c-Kit receptor, was able to induce factor-dependent transformation when overexpressed in NIH/3T3 cells. With the cloning of SLF it was shown that the full length murine c-Kit receptor could also induce factor-dependent transformation (Alexander *et al.*, 1991). The data for human AML described above suggested that overexpression of this receptor may play a role in this disease. This chapter therefore investigates the transforming potential and ligand-dependence of the c-*kit* proto-oncogene product ectopically expressed in NIH/3T3 cells.

RESULTS

4.2 *Overexpression of murine c-kit causes transformation of NIH/3T3 fibroblast cells.*

Ectopic expression of murine c-*kit* in NIH/3T3 cells was achieved by retroviral infection. The cDNA spanning the entire coding region of murine *c-kit*, (Qui *et al.*, 1988) was cloned by Dr. Tom Gonda (Hanson Centre for Cancer Research) from fetal murine brain (as described in section 2.8.1). C-*kit* cDNA was inserted between the Xho I - Hind III sites of the retroviral vector pZen*neo* as shown in Figure 4.2.1. The retroviral construct, designated pZen(mu*kit*) was transfected into the virus packaging cell line, ψ2. NIH/3T3 cells were subsequently infected with viral supernatant collected from a $G418^R$ high titre, pZen(mu*kit*) ψ2 producing clone, X36 (provided by Dr. T. Gonda), and the resultant infectants were designated NIH(mu*kit*). NIH/3T3 cells infected with viral supernatant collected from a pool of ψ2 cells transfected with pZen*neo* were used as a negative control, NIH(*neo*). Expression of the c-Kit receptor by the $G418^R$ NIH(mu*kit*) pool was examined by indirect immunofluorescence (IIF) (see section 2.2.2) using a rabbit polyclonal antiserum to murine c-Kit (provided by Dr. P. Besmer, Memorial Sloan-Kettering Cancer Centre, New York) (Figure 4.2.2 A) and also by northern blot analysis (see section 2.7.5) (Figure 4.2.2 B). As described in Figure 4.2.1 two transcripts of 7 and 6.6 kb were expected, however only the unspliced form was detectable by northern blot analysis. To determine whether the overexpressed normal c-Kit receptor possessed oncogenic potential, the pool of NIH(mu*kit*)

Figure 4.2.1: Composition of the pZen(mu*kit*) retroviral vector. cDNA encoding the entire coding sequence of murine c-*kit* (Qui *et al*, 1988) was inserted between the Xho I-Hind III sites of the pZen*neo* vector. Derivation of the pZen*neo* which contains a 5' LTR from the Moloney-murine leukaemia virus (Mo-MuLV) and a 3' LTR containing R and U5 sequences obtained from the myeloproliferative sarcoma virus (MPSV) as has been described by Johnson *et al.*, (1989). The vector also contained the neomycin gene driven by the thymidine kinase (TK) promoter. Splice donor (SD) and splice acceptor (SA) sites are indicated by arrows. The expected transcripts and their sizes, including the c-*kit* gene, are shown.

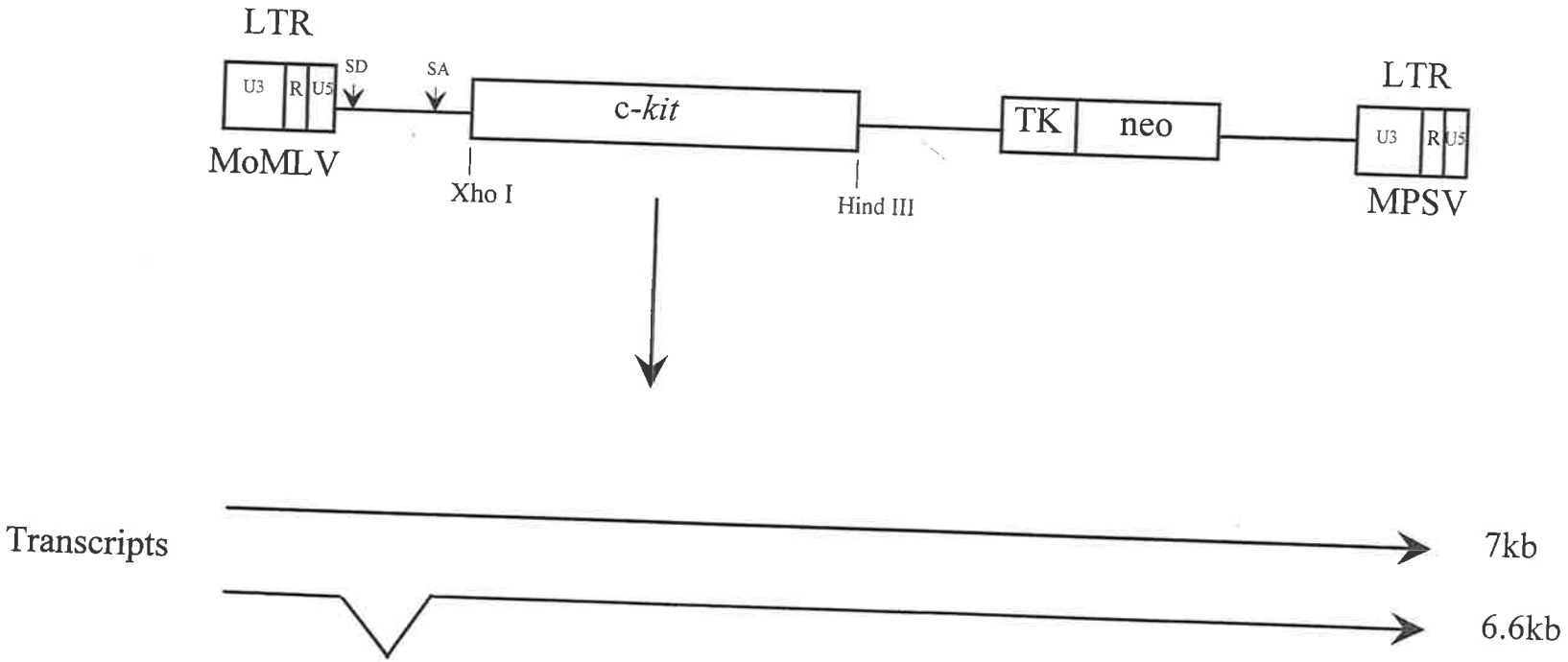
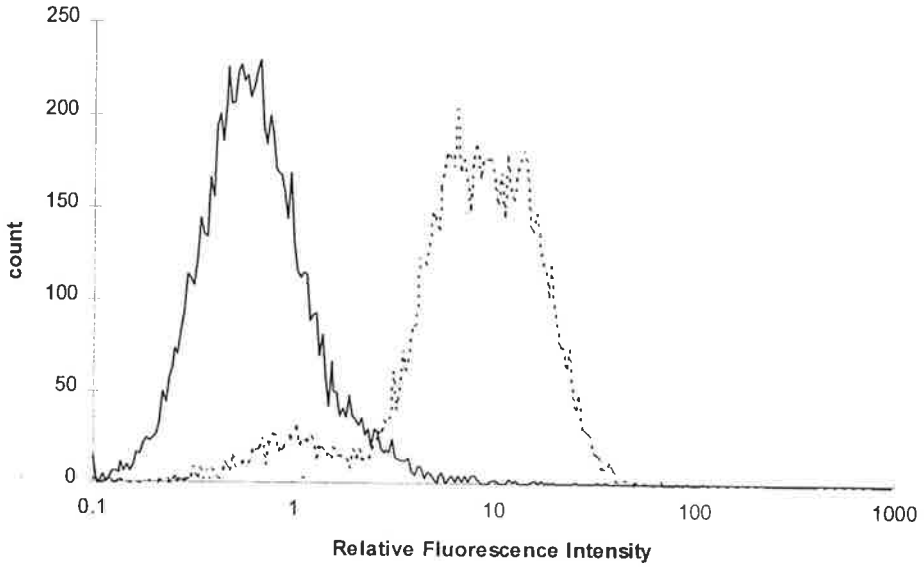
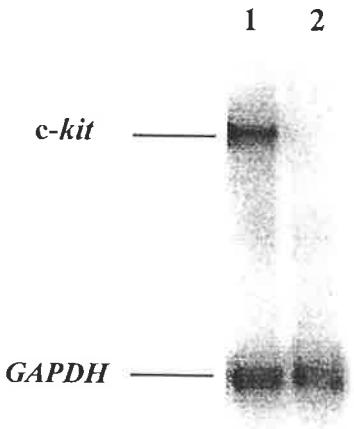

LTR
U3
R
U5
SD
SA
c-kit
TK
neo
LTR
U3
R
U5
MoMLV
Xho I
Hind III
MPSV
Transcripts
7kb
6.6kb

Figure 4.2.2: Expression of c-Kit surface protein and mRNA by the NIH(mu*kit*) infectants. A. Cells were labelled with a polyclonal rabbit antiserum to murine c-Kit and binding was detected by a second stage sheep anti-rabbit fluoresceinated Ig conjugate. The figure illustrates flow cytometry histograms of the NIH(*neo*) (—) and NIH(mu*kit*) (·····) infectants. **B**. 2μg of poly A^+ selected mRNA from NIH(mu*kit*) (lane 1) and NIH(*neo*) (lane 2) were analysed by agarose gel electrophoresis and northern blot hybridisation. Hybridisation was performed using a full length murine c-*kit* probe and a 780 bp human *GAPDH* fragment labelled with ^{32}P. *GAPDH* signals were used as a control for comparing sample loading. Signals visualised as described in section 2.7.5 using a Molecular Dynamics PhosphorImager.


250
200
150
100
50
0
count
0.1
1
10
100
1000
Relative Fluorescence Intensity


1
2
c-kit
GAPDH

infectants was assayed in the classical *in vitro* transformation assays in the absence or presence of exogenously added recombinant murine SLF (mSLF).

As shown in Table 4.2.1 the NIH(mu*kit*) cells, in contrast to the control NIH(*neo*) cells, had the ability to grow at low density, in low concentrations (1%) of fetal bovine serum (FBS), in the presence or absence of exogenously added mSLF. The presence of mSLF enhanced the ability of the cells to grow in low serum conditions (39.9% plating efficiency), compared to those which lacked mSLF (27%). There was also a discernible morphological change in the colonies obtained in the presence of mSLF (Figure 4.2.3). The colonies that grew in 1% serum + mSLF consisted of refractile rounded cells that were less adherent to the substratum. These cells also piled up on top of one another in a disorganised array, (Figure 4.2.3, bottom of page) compared to the flat, more adherent dull colonies which grew in 1% serum - mSLF (Figure 4.2.3, top of page). This difference in appearance between the colonies obtained in the absence and presence of mSLF was also evident in those colonies arising in 10% serum, however it was less dramatic (data not shown). The piling up of rounded cells represents focus formation, a result of the loss of contact inhibition, another characteristic indicative of transformation. This loss of contact inhibition also resulted in more dense monolayers of NIH(mu*kit*) cells at confluence in the presence of mSLF (Figure 4.2.4, bottom of page), compared to those cultured in the absence of mSLF or the NIH(*neo*) control cells (Figure 4.2.4, middle and top of page).

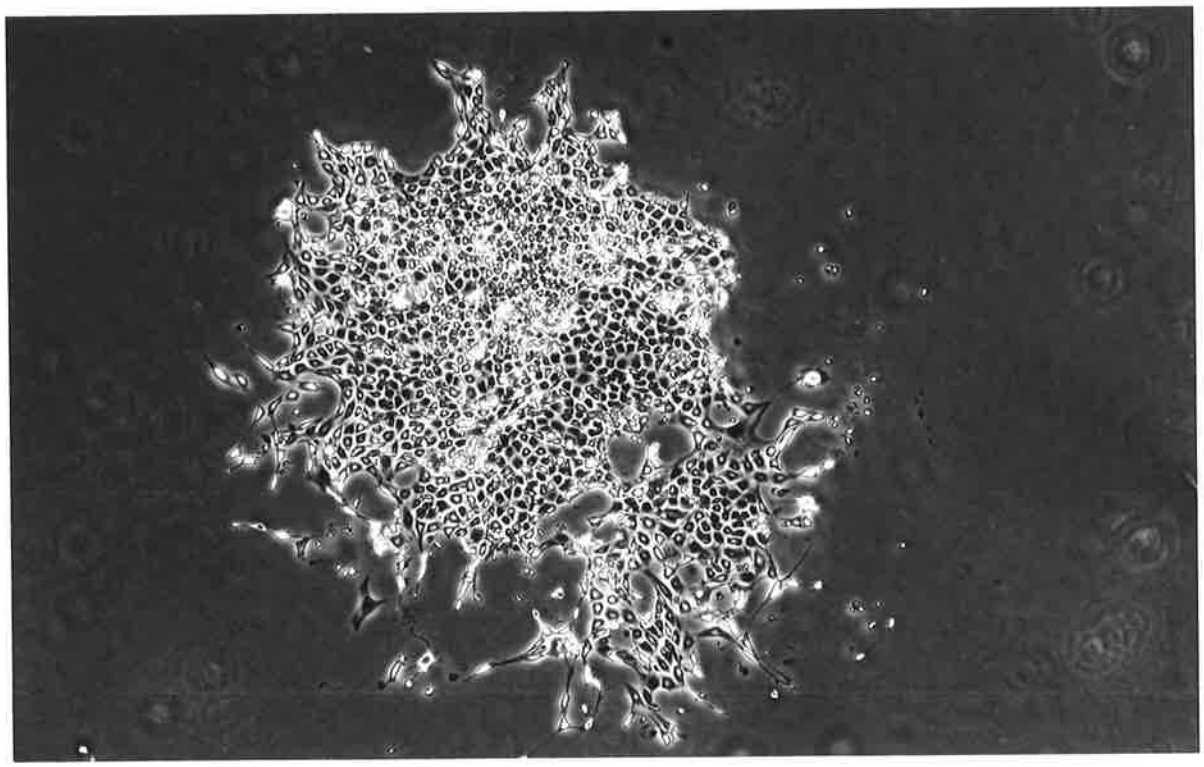
Another characteristic indicative of transformation is the ability to grow in soft agar, indicating the loss of anchorage dependence (see section 2.9.3 for method). NIH(mu*kit*) cells were able to produce colonies in soft agar in a ligand-dependent manner, colony number and size increasing with increasing concentrations of mSLF. The average plating efficiency ranged from 1.4% in the presence of 10 ng/ml mSLF to 11% in the presence of 200 ng/ml mSLF. In the absence of exogenous mSLF colonies were also produced at an average plating efficiency of 1.2%. NIH(*neo*) cells did not display anchorage independence. These frequencies were routinely obtained in repeated experiments and Figure 4.2.5.A. displays the results of a typical assay. The low frequency of colonies produced in the absence of added factor is possibly due to the endogenous production of SLF by the NIH/3T3 cells (described in Chapter 3) thereby

Table 4.2.1: Assay for the growth in 1% fetal bovine serum (FBS) of NIH(mu*kit*) pool infectants.

infectant	Plating efficiency in 1 % FBS (%)
NIH(*neo*) + SLF	0.0
NIH(mu*kit*) - SLF	27.0
NIH(mu*kit*) + SLF	39.7

10^2 cells were plated in medium containing 1% or 10% FBS in the presence or absence of 100 ng/ml mSLF. The plating efficiency in 1% FBS is relative to the number of colonies arising in 10% FBS under the same culture conditions.

Figure 4.2.3: Morphology of NIH(mu*kit*) infectants grown in low serum. 10^2 NIH(mu*kit*) cells were plated in 1% FBS in the absence (top photograph) or presence (bottom photograph) of 100 ng/ml mSLF. Cultures were fed twice weekly. Photographs of typical colonies were taken 10 days after initiation of cultures, under phase contrast optics at 4x magnification.



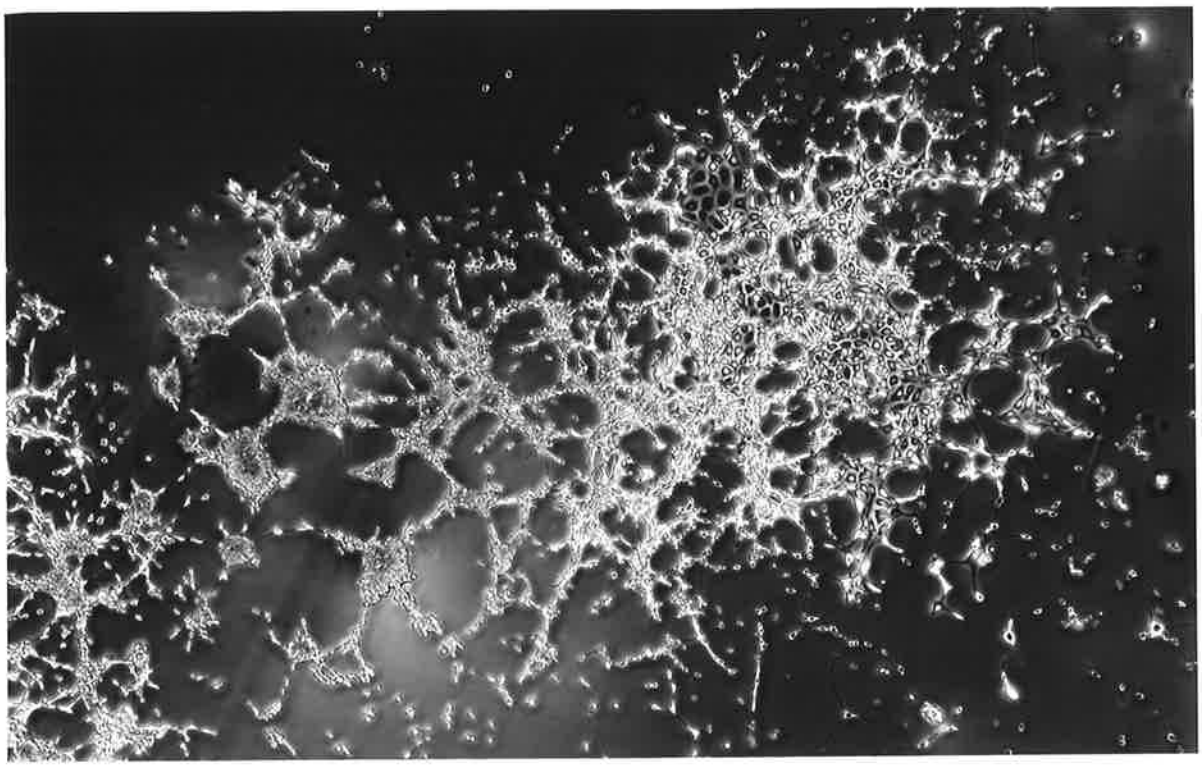
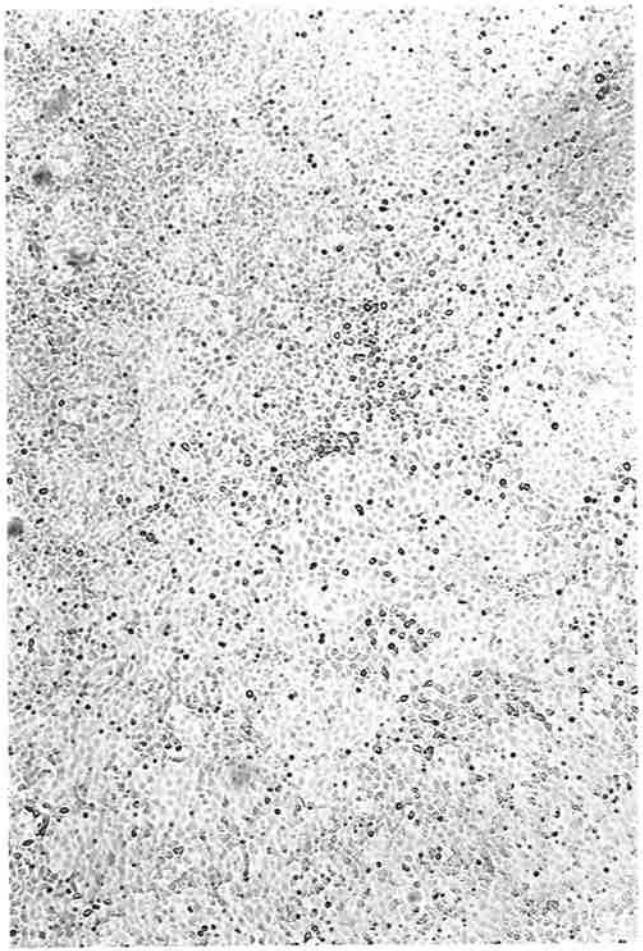
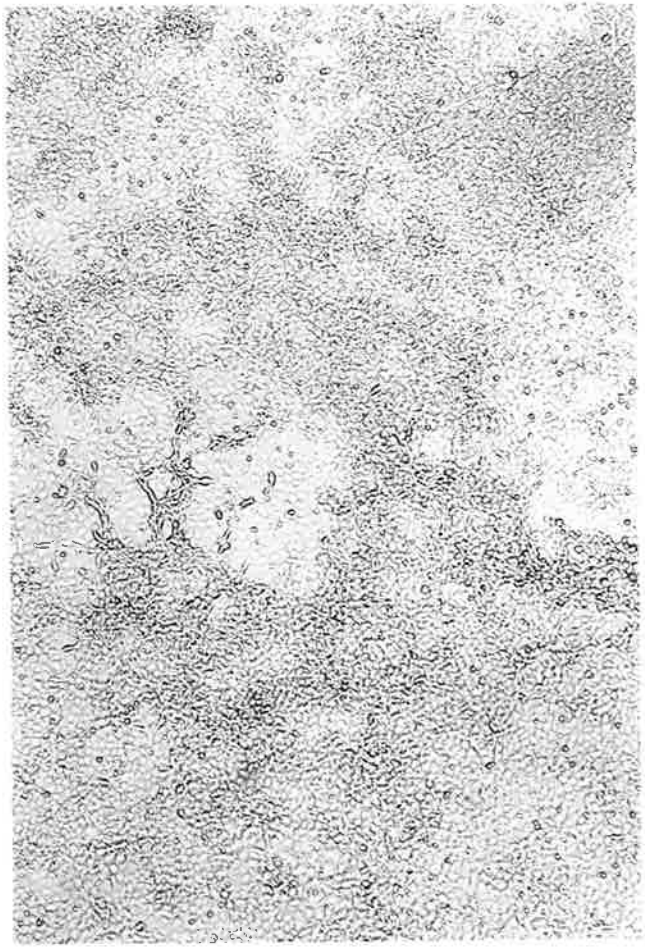


Figure 4.2.4: Morphology of NIH(mu*kit*) cell monolayers. NIH(mu*kit*) cells were plated in the absence (middle photograph) and presence (bottom photograph) of 100 ng/ml mSLF and NIH(*neo*) cells in the presence of 100 ng/ml mSLF (top photograph). Photographs taken at 4x magnification of typical regions of the monolayers.





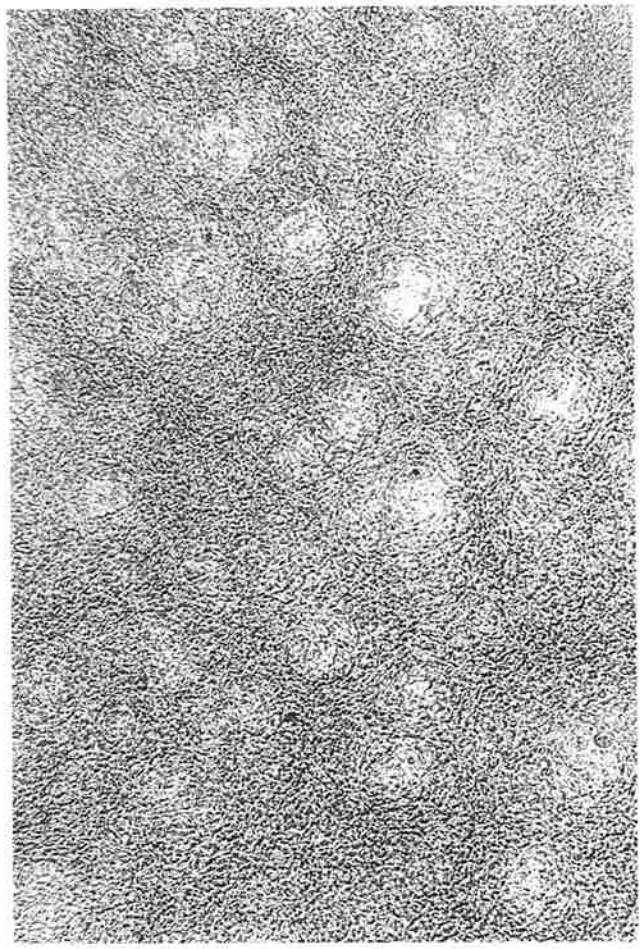


Figure 4.2.5: Anchorage independent growth of the NIH(mu*kit*) pool of infectants in soft agar. (**A**) 2 x 10^3 cells were plated in 0.33% semi-solid agar in the presence of increasing concentrations of mSLF as indicated. Colonies consisting of >50 cells were scored microscopically after 14 days. * = NIH(*neo*) control cells which produced a negligible number of colonies 1 ± 0. Colony numbers represented as the mean ± S.E.M of quadruplicate replicates.

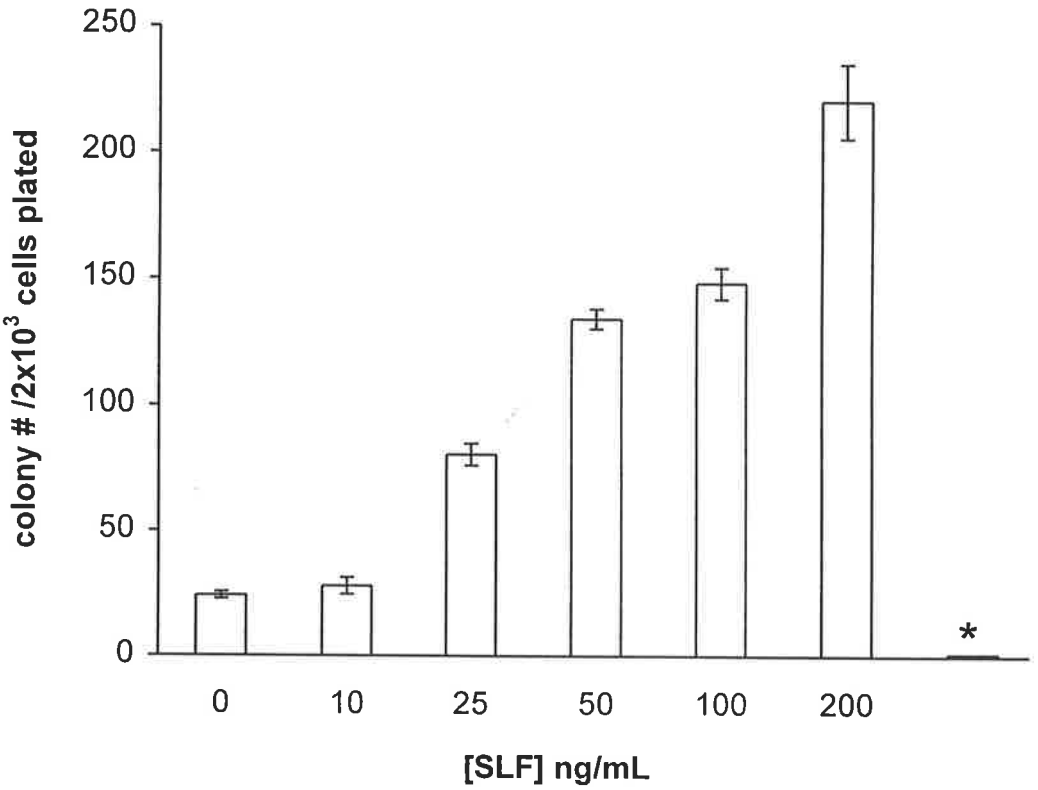
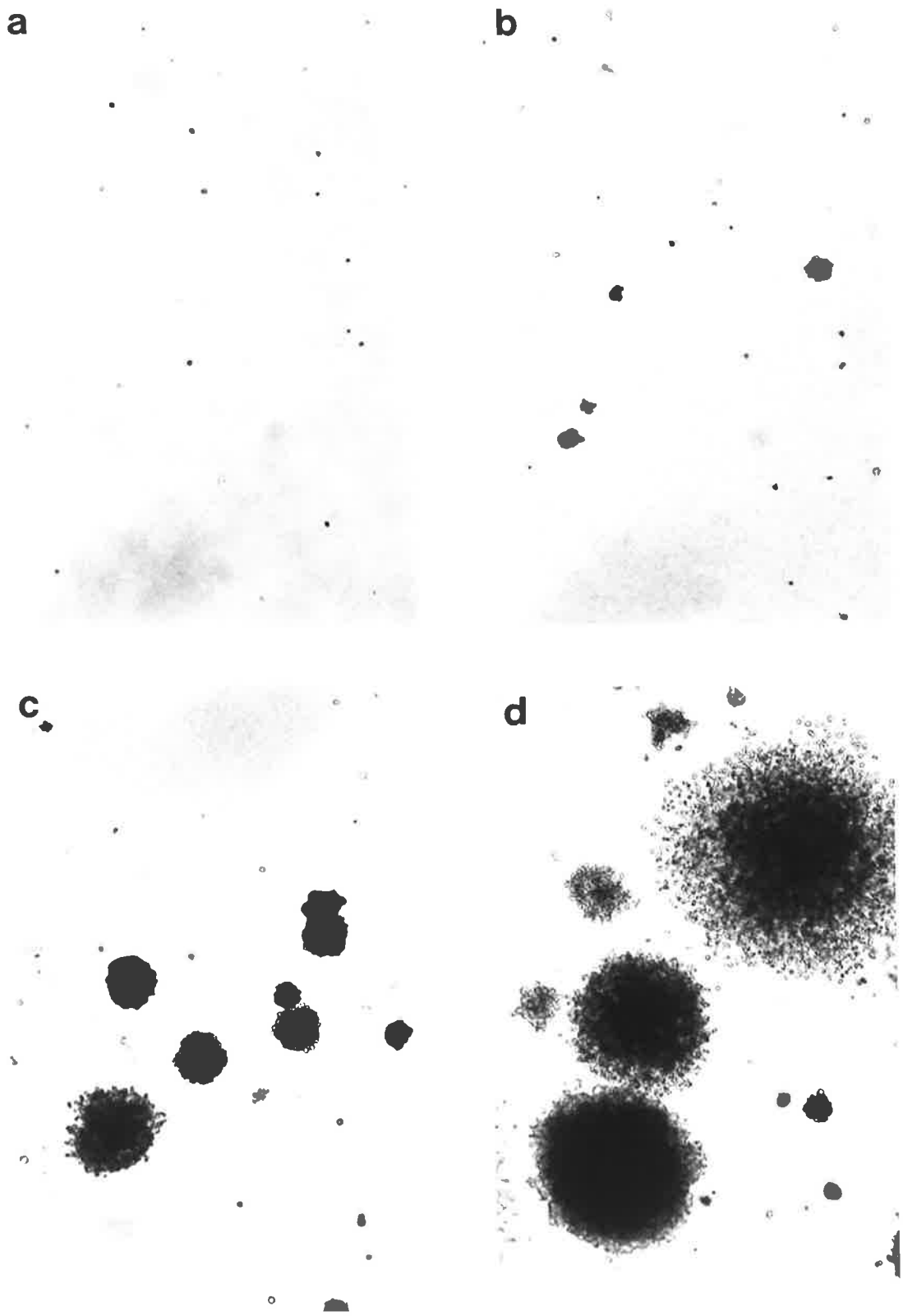

250
200
150
100
50
0
colony # /2x10^3 cells plated
*
0
10
25
50
100
200
[SLF] ng/mL

Figure 4.2.5: Anchorage independent growth of the NIH(mu*kit*) pool of infectants in soft agar. (**B**) Morphology of the colonies produced by (**a**) NIH(*neo*) + 100 ng/ml mSLF, (**b**) NIH(mu*kit*) - mSLF, (**c**) NIH(mu*kit*) + 50 ng/ml mSLF, (**d**) NIH(mu*kit*) + 200 ng/ml mSLF. Photographs taken of representative areas of the plates on day 14 at 4x magnification.

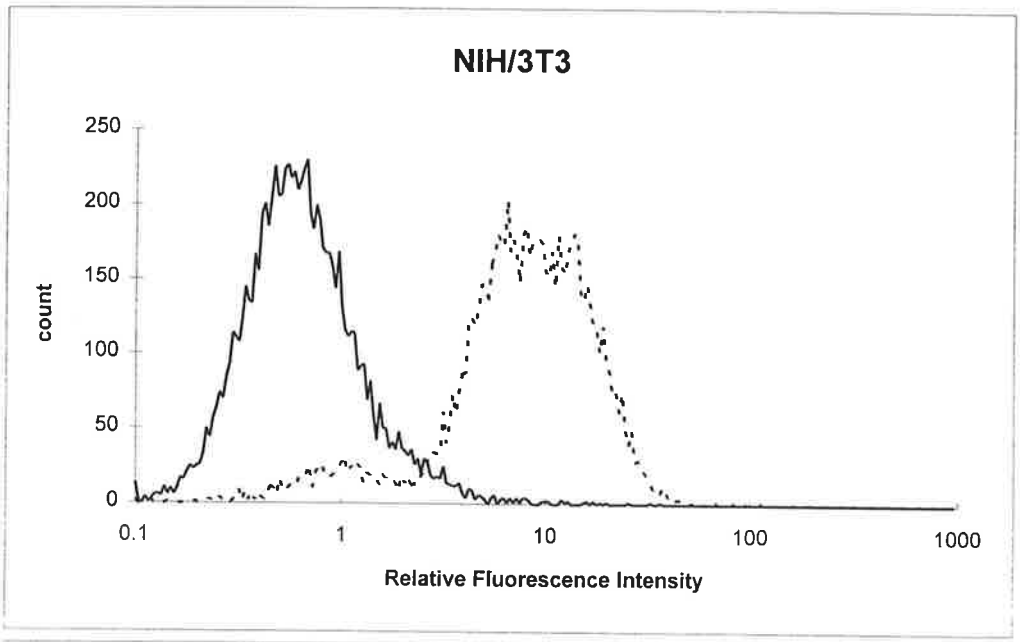

a
b
c
d

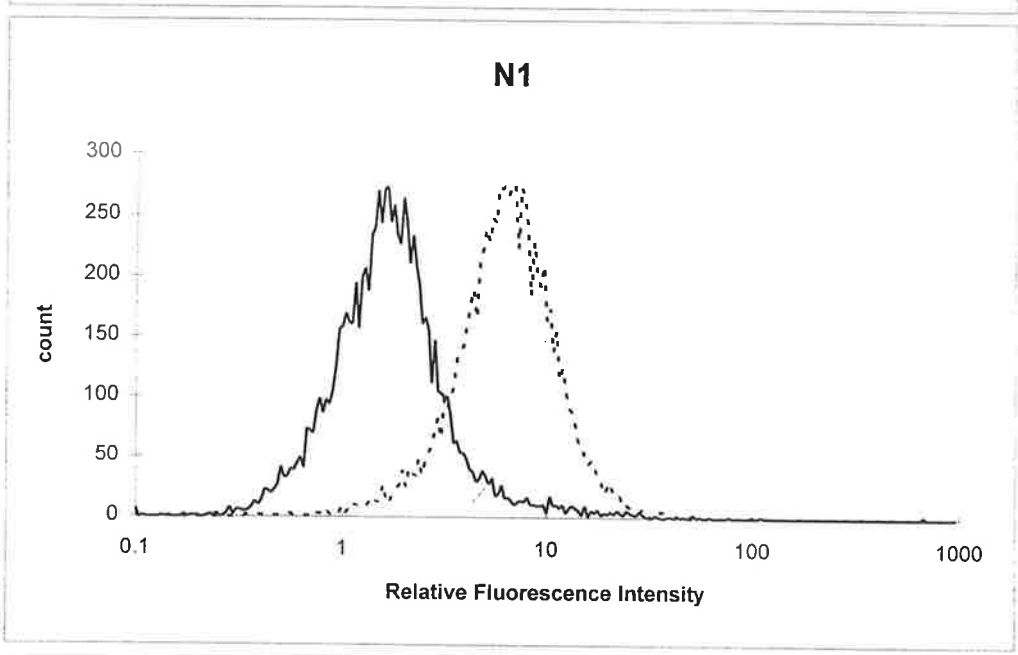
setting up an autocrine loop. It is also possible that within the pool of NIH(mu*kit*) cells that there are some cells which have the ability to show anchorage independence in low levels of SLF due to high levels of c-Kit expression. Alternatively, anchorage independence in the absence of exogenous mSLF may be due to an event unrelated to the introduction of c-*kit* resulting in spontaneous transformation. These possibilities will be addressed throughout the remainder of this chapter.

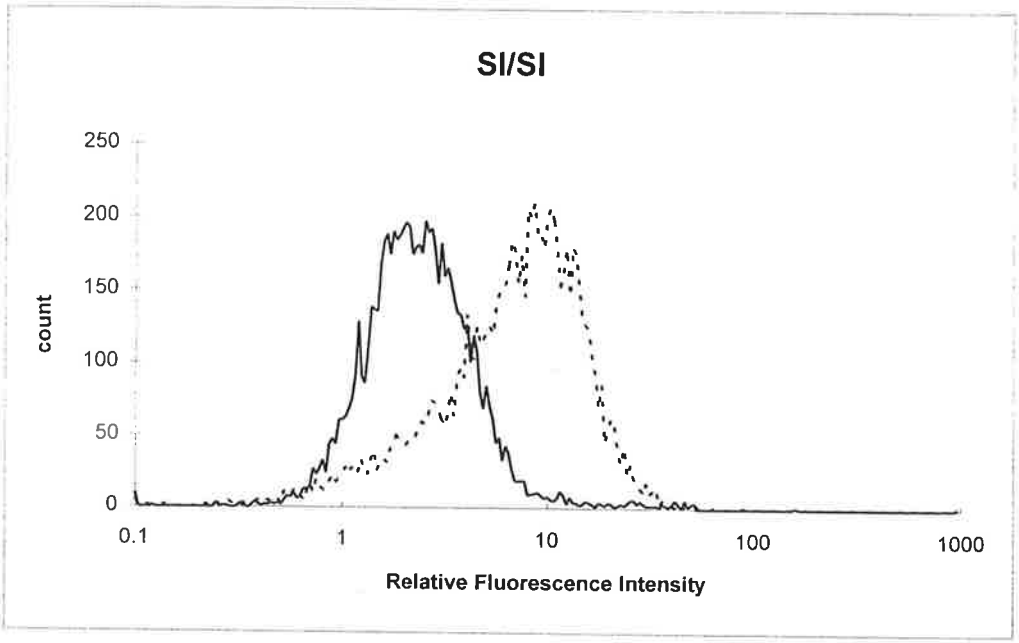
4.3 Does transformation occur in the absence of endogenous SLF?

In order to investigate the transforming potential of c-Kit in the absence of endogenous SLF production, *Sl/Sl*-3T3 ($WCB6F_1$/3T3) cells were infected with viral supernatant obtained from the ψ2 clone, X36. A population of $G418^R$ *Sl/Sl*(mu*kit)* cells expressing a similar level of cell surface c-Kit to that expressed by the NIH(mu*kit*) infectants were obtained by fluorescence-activated cell sorting (see section 2.2.3 for method). As a positive control, cells of the same genetic background as *Sl/Sl*-3T3 cells, N1-3T3 cells, which produce both soluble and membrane bound SLF, as described in chapter 3 were also infected. *Sl/Sl* and N1-3T3 cells were also infected with viral supernatant produced by the ψ2 pZen*neo* packaging line as negative controls. The levels of c-Kit surface protein expressed by these infectants were analysed by IIF and compared to the NIH(mu*kit*) cell population. Both N1 and *Sl/Sl*-3T3(*neo*) cells gave higher background binding to the α-c-Kit polyclonal antibody than NIH(*neo*) cells, however N1 and Sl/Sl(mu*kit*) infectants displayed comparable levels to each other and this level of c-Kit expression lay within the range seen in the pool of NIH(mu*kit*) cells (Figure 4.3.1). *Sl/Sl*(mu*kit*) cells did not display anchorage independence, even in the presence of high concentrations of mSLF or when plated at a 10 fold higher density than NIH(mu*kit*) cells, and N1 infectants produced a negligible number of colonies in the presence of 100 ng/ml mSLF (plating efficiency of 0.44%) and no colonies in the absence of mSLF. This suggests that *Sl/Sl* and N1-3T3 cells are not permissive to transformation. It is known that multiple events are required to induce a transformed phenotype (Land *et al.*, 1983a); it is possible that these cells have not undergone the same events during immortalisation as NIH/3T3 cells prior to the introduction of c-*kit*.

Figure 4.3.1: Expression of cell surface c-Kit protein in murine fibroblasts infected with the pZen(mu*kit*) retrovirus. (A) Fluorescence histograms illustrating the surface expression of c-Kit detected by indirect immunofluorescence as described in Figure 4.2.2 on both pZen(*neo*) (——) and pZen(mu*kit*) (·····) fibroblast infectants NIH/3T3, N1(WCB6F$_1$/3T3) and *Sl/Sl* (WCB6F$_1$/3T3) cells.


NIH/3T3
250
200
150
100
50
0
count
0.1
1
10
100
1000
Relative Fluorescence Intensity


N1
300
250
200
150
100
50
0
count
0.1
1
10
100
1000
Relative Fluorescence Intensity


SI/SI
250
200
150
100
50
0
count
0.1
1
10
100
1000
Relative Fluorescence Intensity

Since transformation of the SLF negative, *Sl/Sl*(mu*kit*) infectants could not be induced, attempts were made to block the action of endogenously produced SLF by NIH(mu*kit*) cells. An antagonistic α-c-Kit mAb, ACK2, (Ogawa *et al.*, 1991) and an α-SLF polyclonal antiserum were independently used. The activity of these antibodies was first verified by assaying their ability to block the proliferation of FDC-P1(SLF) cells maintained in 10 ng/ml mSLF. As shown in Table 4.3.1. 50 ng/ml ACK2 was able to completely inhibit the proliferation of the cells grown in mSLF, however the α-SLF antiserum at 10 μg/ml was only able to inhibit proliferation by approximately 88%. Neither antibody had an effect on the proliferation of cells grown in 10 U/ml of recombinant murine GM-CSF. Inclusion of these antibodies in the anchorage independence soft agar colony assay did not inhibit colony formation of cells plated in the absence of exogenous mSLF. However, ACK2 and α-SLF were able to inhibit the formation of colonies produced in the presence of 100 ng/ml mSLF by 90% and 66%, respectively, while the isotype-matched negative control antibodies had no effect (Figure 4.3.2). Interestingly, in the presence of mSLF, the number of colonies resistant to ACK2 inhibition (representing ~1.6% of the cells plated) was reproducibly identical to those produced by cells plated in the absence of mSLF irrespective of whether they had received antibody. The lack of equivalent inhibition by the α-SLF antibody compared to that achieved by ACK2 was not surprising since it was demonstrated in Table 4.3.1 (and also described by the manufacture, Genzyme) that this antibody was unable to totally inhibit mSLF-dependent proliferation.

4.4 *Analysis of clones able to grow in the absence of exogenously added SLF.*

Since the colonies produced in the absence of exogenous mSLF were resistant to the inhibitory action of ACK2 and α-SLF antisera, it was possible that these cells did not express functional c-Kit receptors. In order to investigate this, 5 colonies were plucked from dishes that did not receive mSLF. The colonies plucked were selected based on size; ranging from very large (colony A, ~2000 cells) progressively getting smaller (B ~1000 cells, C ~500 cells and D ~100 cells), the smallest colony selected consisting of a cluster of 10 cells (colony E). These were expanded in liquid culture for two weeks. Cells were analysed for their levels of

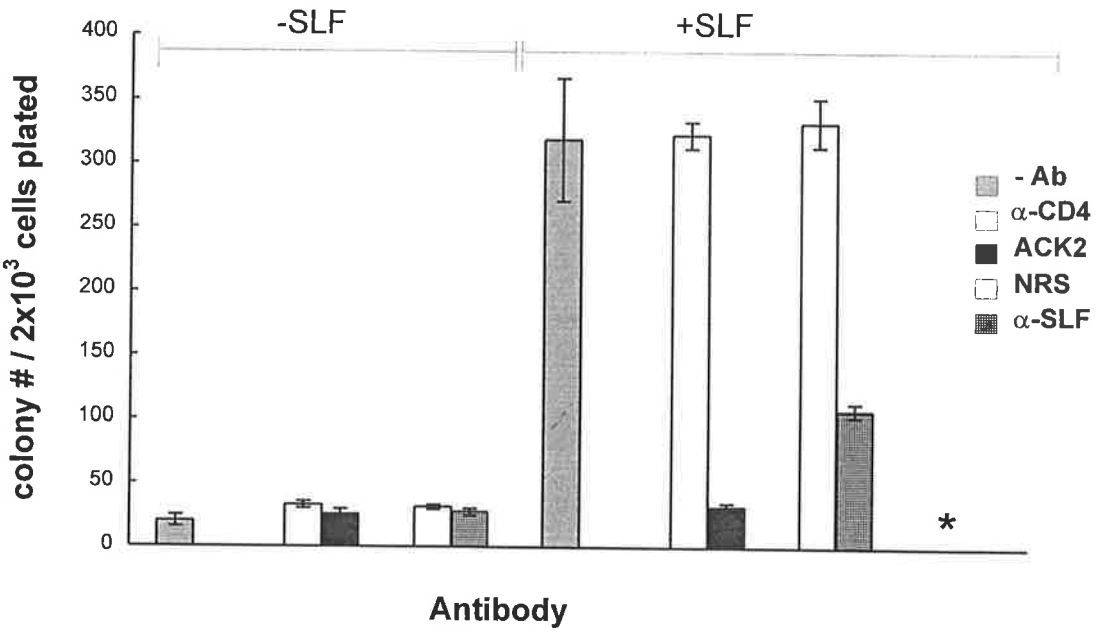
Table 4.3.1: Inhibition of mSLF-dependent proliferation of FDC-P1(SLF) cells.

ACK2 (μg/ml)	α-SLF (μg/ml)	mSLF	mGM-CSF	^{3}H-thymidine uptake (cpm ± S.E.M)
-	-	-	-	150.7 ± 0
-	-	+	-	13,957.5 ± 765
0.05	-	+	-	129.7 ±16
1.00	-	+	-	159.0 ± 15
-	1.00	+	-	11,736.3 ±1,955
-	10.00	+	-	1,694.9 ± 266
-	-	-	+	188,728.3 ± 8,765
0.05	-	-	+	175,787.3 ± 6,680
1.00	-	-	+	188,457.3 ± 11,174
-	1.00	-	+	179,772.7 ± 14,896
-	10.00	-	+	185,507.0 ± 9,211

10^4 FDC-P1(SLF) cells were cultured in the presence of 10 ng/ml mSLF or 10 U/ml mGM-CSF with varying concentrations of the mAb, ACK2, or the α-SLF antiserum as indicated. Cultures were incubated for 48 hours at 37°C in 5% CO_2, then pulsed with 1μCi ^{3}H-thymidine for 18 hours, harvested and counted by liquid scintillation. Data represented as the mean ± S.E.M. from triplicate wells.

Figure 4.3.2: Inhibition of anchorage independence by ACK2 and α-SLF antibodies. 2 x 10^3 NIH(mu*kit*) and NIH(*neo*) cells were plated in 0.33% semi-solid agar in the presence or absence of 100 ng/ml mSLF. Where indicated 5 μg/ml ACK2 or the equivalent isotype-matched (IgG_{2A}) negative control antibody, α-CD4, or 20 μg/ml of the polyclonal rabbit α-SLF antiserum or non-immune rabbit serum were also included. Colony numbers represented as the mean ± S.E.M of quadruplicate replicates.

* NIH(*neo*) cells did not produce any colonies.

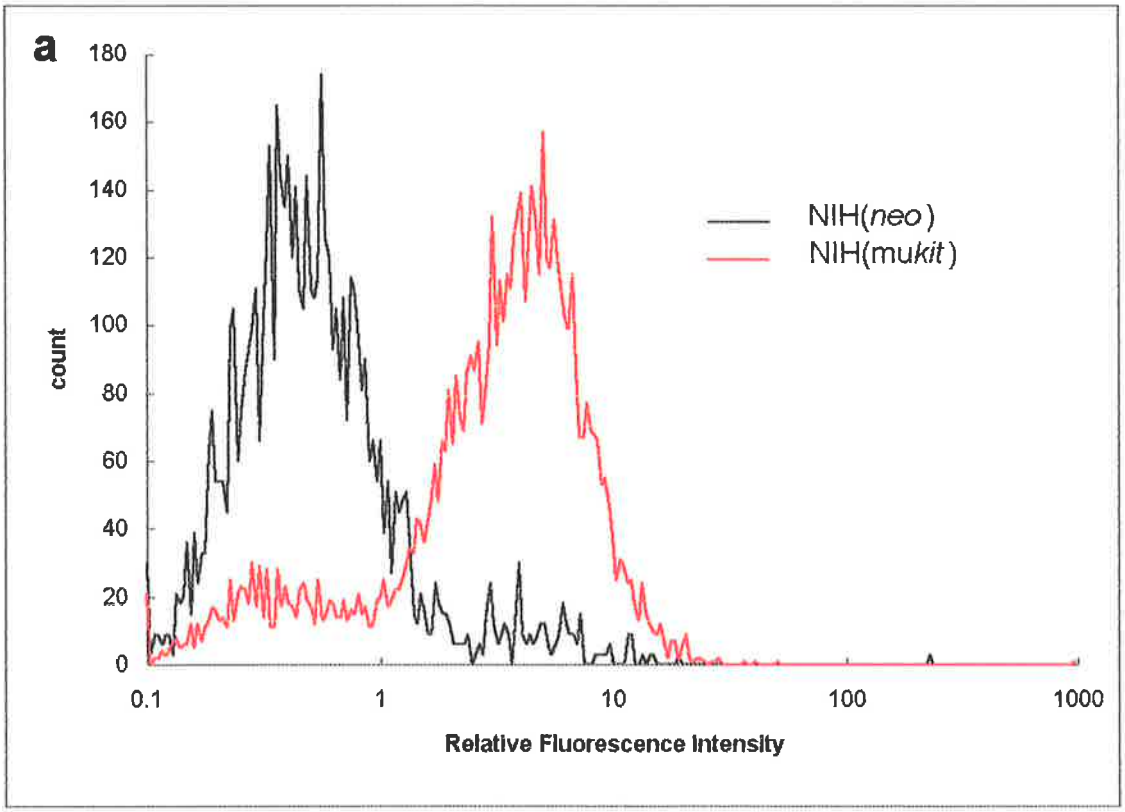

-SLF
+SLF
400
350
300
250
200
150
100
50
0
colony # / 2x10^3 cells plated
- Ab
α-CD4
ACK2
NRS
α-SLF
*
Antibody

c-Kit protein and mRNA expression. Resistance to ACK2 or α-SLF inhibition could not be attributed to lack of c-Kit expression or gross alteration in the size of the c-*kit* mRNA transcript (Figure 4.4.1A, B). The level of c-Kit expressed by the clones A-D were similar, yielding an average relative mean fluorescence intensity (mfi) of 5.8 ± 0.4, with E expressing a slightly lower level of surface c-Kit protein, mfi 4.3 (refer to Table 4.4.1). These clones represented medium levels of c-Kit expression within the NIH(mu*kit*) pool (Figure 4.4.1A). The levels of c-*kit* mRNA, as determined by PhosphorImager analysis using the level of *GAPDH* expression as a comparative loading control, also demonstrated that clones A-C expressed similar levels of c-*kit* with D and E expressing approximately 2 fold lower levels (Figure 4.4.1B, also Table 4.4.1). Thus the size of the colonies originally plucked did not depend on c-Kit expression levels. To determine whether the c-Kit receptor expressed by these cells was responsive to mSLF, the clones were replated in soft agar in the presence or absence of mSLF. The results displayed in Table 4.4.1 show that the clones possessed functional c-Kit receptors as indicated by their responsiveness to 100 ng/ml mSLF. Colonies were produced at an average plating efficiency ranging from 0.5% to 3.76% in the absence of mSLF and 3% to 10.7% in the presence of mSLF. These frequencies were reconfirmed in a repeat experiment.

4.5 *What level of c-Kit expression is required for transformation?*

To determine what levels of c-Kit expression within the NIH(mu*kit*) population were required to induce transformation, clones were obtained from the pool of infectants by fluorescence-activated cell sorting. Cells were sorted from low, medium and high c-Kit expressing regions of the NIH(mu*kit*) population and clones were then obtained by plating cells from these sorted regions at limiting dilution. The levels of surface c-Kit protein expressed by these clones spanned the entire range of c-Kit seen in the NIH(mu*kit*) pool (6 fold range). Clones were categorised into low, medium, and high c-Kit expressing groups based on distinct changes in mfi, visualised as a histogram peak shift, as portrayed in Figure 4.5.1.A. A 16.8 fold range of c-*kit* mRNA expression was represented by these clones; these levels were also considered when determining the c-Kit expression groups (Figure 4.5.1.B.).

Figure 4.4.1 A: c-Kit surface protein expression in the cells derived from the plucked colonies NIH(mu*kit*)-(A-E). Fluorescence histograms illustrating the surface expression of c-Kit detected by indirect immunofluorescence as described in Figure 4.2.2 on the NIH(*neo*) and NIH(mu*kit*) pools (a) and on the plucked colonies A-E (b).


a
180
160
140
120
100
80
60
40
20
0
count
0.1
1
10
100
1000
Relative Fluorescence Intensity
NIH(neo)
NIH(mukit)

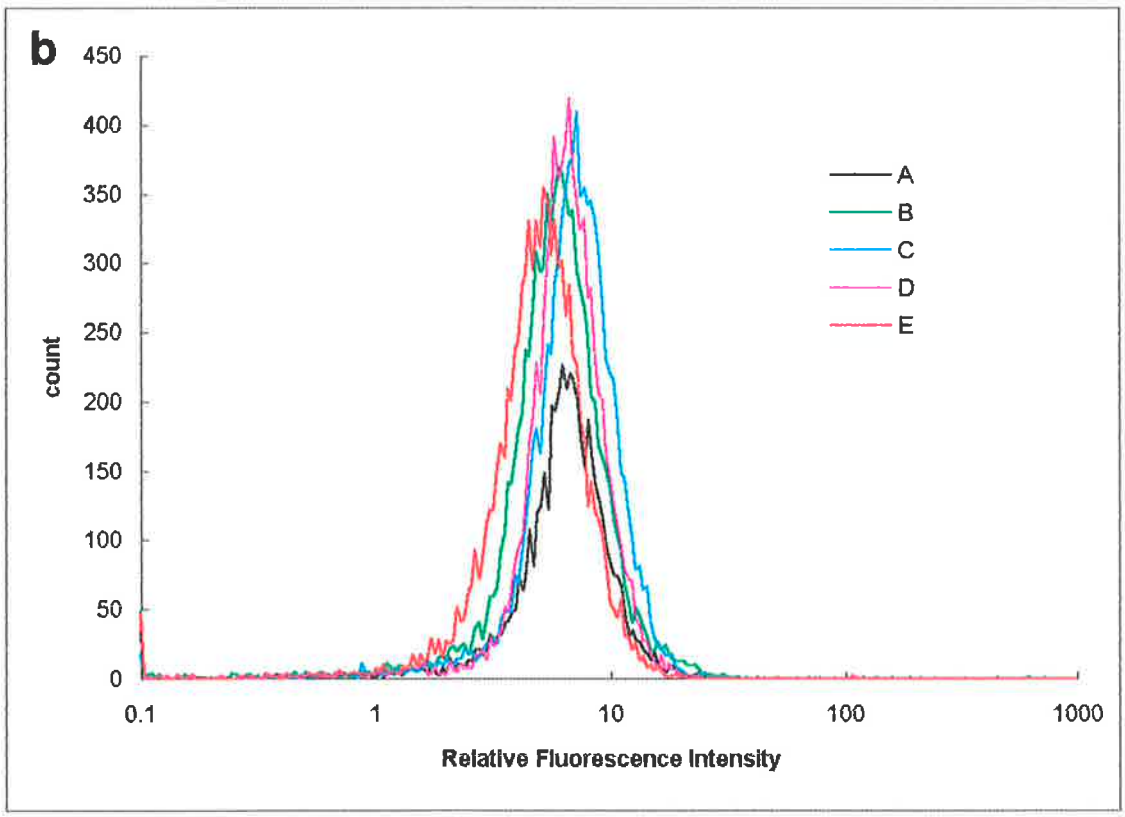
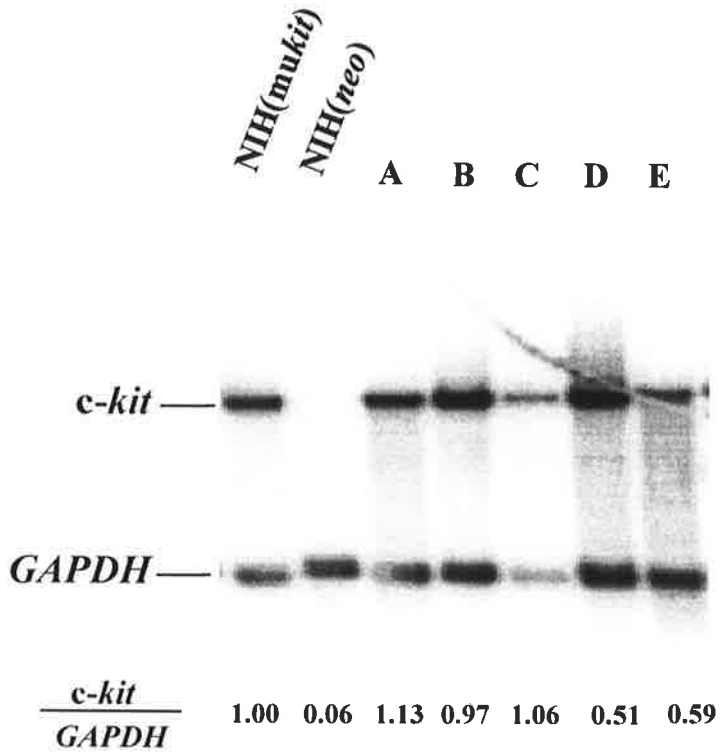

b
450
400
350
300
250
200
150
100
50
0
count
0.1
1
10
100
1000
Relative Fluorescence Intensity
A
B
C
D
E

Figure 4.4.1 B: c-*kit* mRNA expression in the cells derived from the plucked colonies NIH(mu*kit*)-(A-E) Expression of c-*kit* mRNA in NIH(mu*kit*) pool, NIH(*neo*) control cells, NIH(mu*kit*)-A-E detected by Northern blot hybridisation as described for Figure 4.2.2. c-*kit* hybridisation signals were quantitated as described in section 2.7.5 and expressed as c-*kit*/*GAPDH* signal ratios relative to the NIH(mu*kit*) pool.



c-kit / GAPDH	1.00	0.06	1.13	0.97	1.06	0.51	0.59

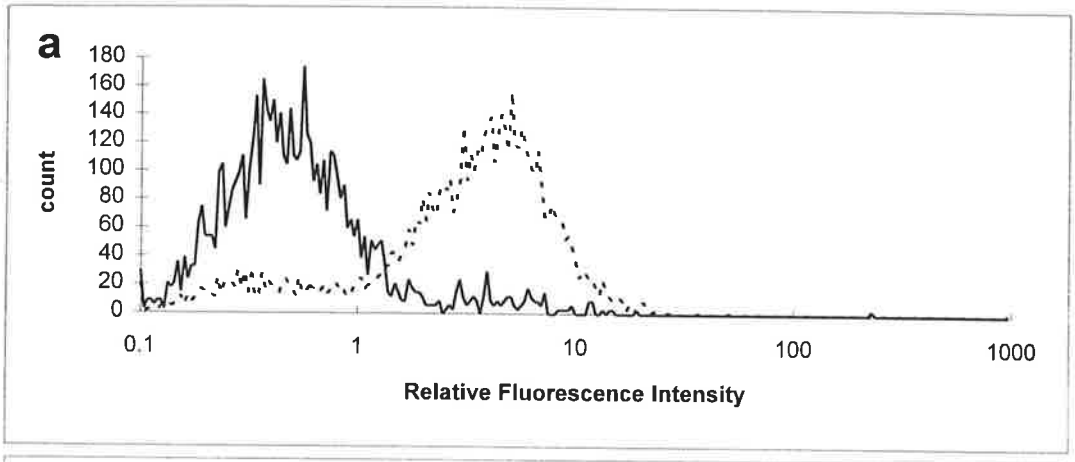
Table 4.4.1: Properties of NIH(mu*kit*)A-E cells.

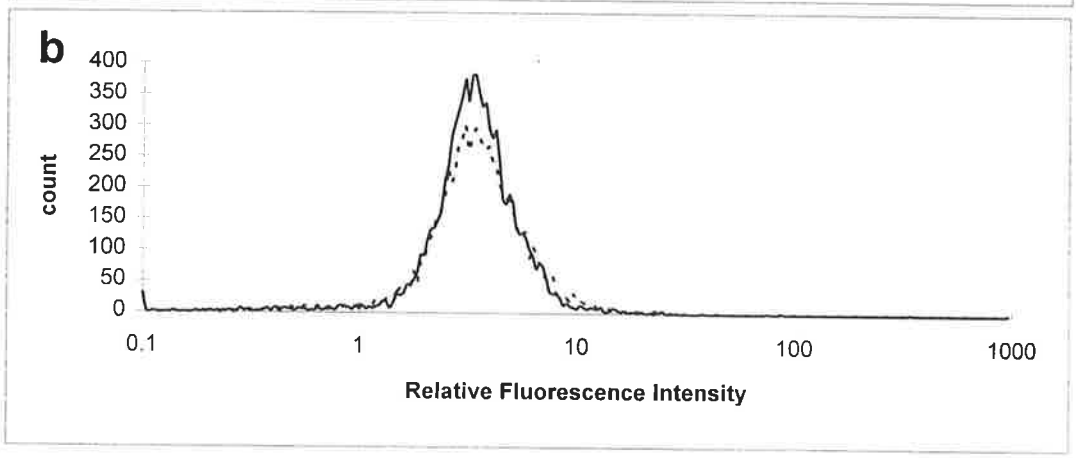
cell	relative mfi	c-*kit* mRNA*	colony # / 10^3 cells∞	
			- mSLF	+ mSLF
NIH(*neo*)	0.0	0.07	0.11±0.08	0.16±0.59
NIH(mu*kit*) pool	2.4	1.00	11.15±0.50	58.46±1.44
NIH(mu*kit*)-A	5.6	1.13	20.30±1.00	107.46±5.25
NIH(mu*kit*)-B	5.3	0.97	8.60±1.60	30.40±2.20
NIH(mukit)-C	6.4	1.06	18.20±1.10	61.86±6.02
NIH(mu*kit*)-D	5.8	0.51	37.60±1.60	105.82±12.05
NIH(mu*kit*)-E	4.3	0.59	5.30±0.70	88.22±10.04

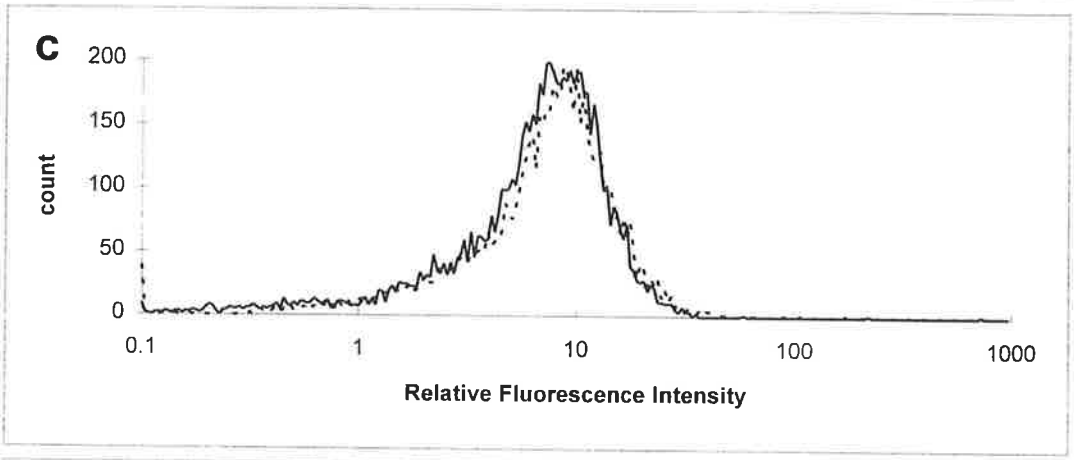
* relative to NIH(mu*kit*) pool

∞ 10^4 cells plated in 0.33% soft agar in the absence or presence of 100 ng/ml mSLF and colonies scored on day 14. Colony number represented as the mean ± S.E.M of quadruplicate replicates.

Figure 4.5.1 A: Expression of c-Kit protein by NIH(mu*kit*) clones categorised into low, medium and high c-Kit expression groups relative to the pool of infectants. Surface expression of c-Kit was detected by indirect immunofluorescence as described in Figure 4.2.2 on (a) NIH(*neo*)(—) and NIH(mu*kit*) (····), (b) low c-Kit expressers; R31 (—) and R49 (····), (c) medium expressers; R33 (—) and R64 (····) and (d) high expressers; R68 (—) and R56 (····).


a
count
180
160
140
120
100
80
60
40
20
0
0.1
1
10
100
1000
Relative Fluorescence Intensity


b
count
400
350
300
250
200
150
100
50
0
0.1
1
10
100
1000
Relative Fluorescence Intensity


c
count
200
150
100
50
0
0.1
1
10
100
1000
Relative Fluorescence Intensity

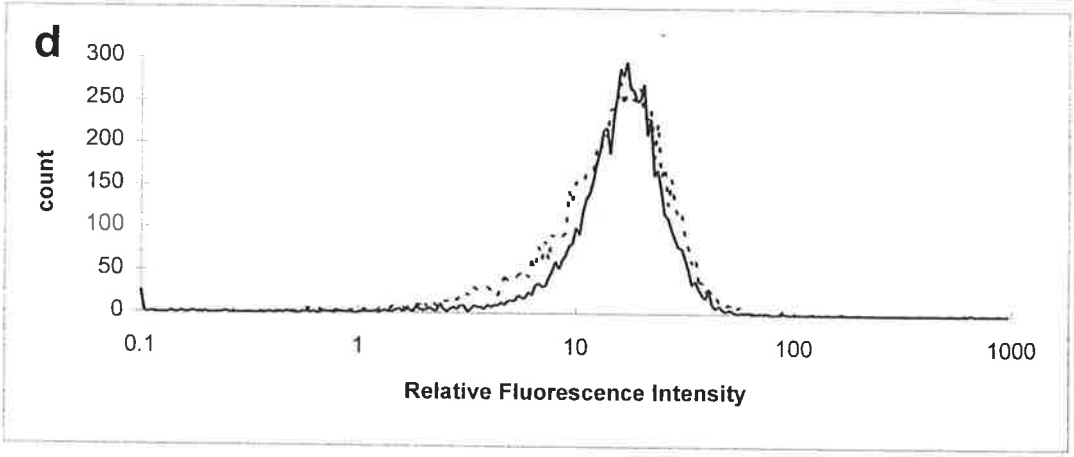
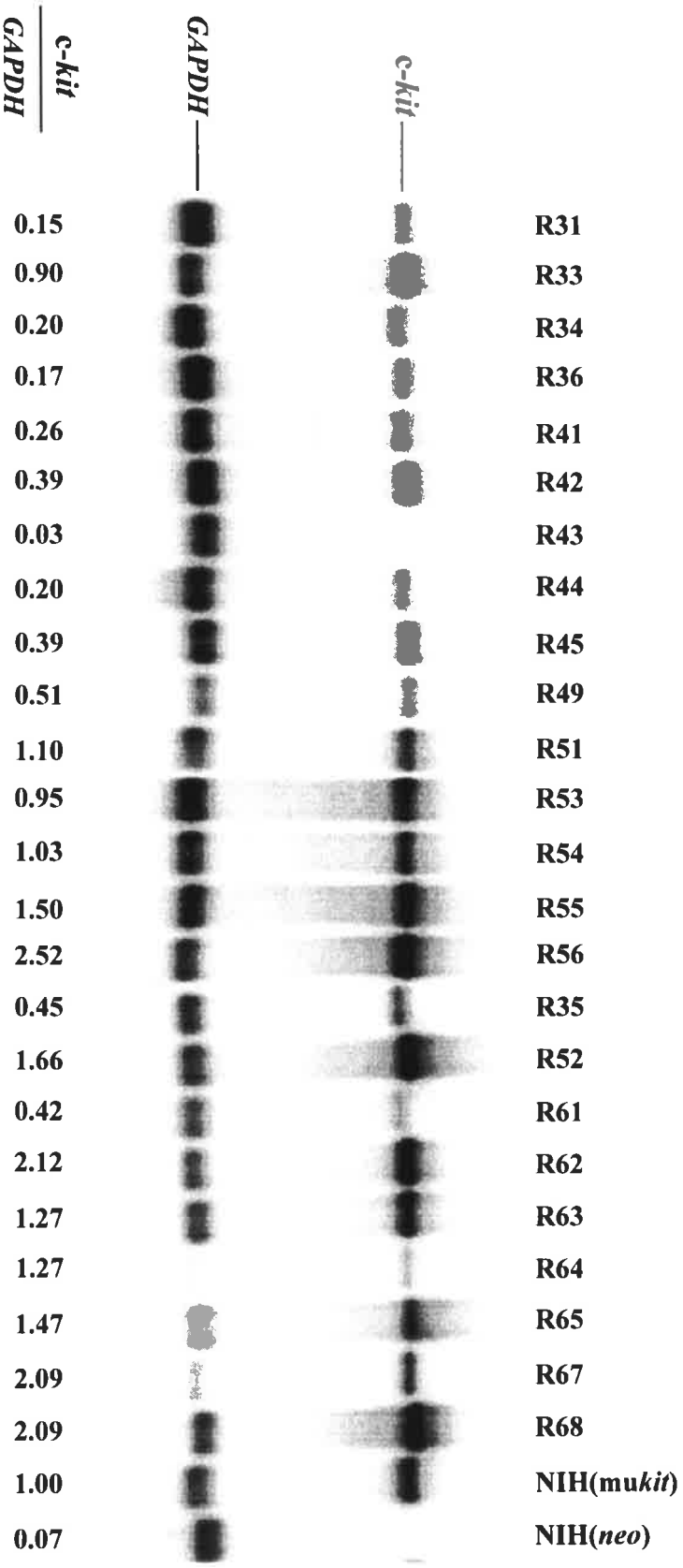

d
count
300
250
200
150
100
50
0
0.1
1
10
100
1000
Relative Fluorescence Intensity

Figure 4.5.1 B: Expression of c-*kit* mRNA by NIH(mu*kit*) clones relative to the pool of infectants. c-*kit* mRNA expression was detected by northern blot hybridisation as described in Figure 4.2.2 and the c-*kit* signal expressed as c-*kit*/*GAPDH* signal ratio relative to the NIH(mu*kit*) pool as described in Figure 4.4.1.


c-kit / GAPDH
GAPDH
c-kit
0.15 R31
0.90 R33
0.20 R34
0.17 R36
0.26 R41
0.39 R42
0.03 R43
0.20 R44
0.39 R45
0.51 R49
1.10 R51
0.95 R53
1.03 R54
1.50 R55
2.52 R56
0.45 R35
1.66 R52
0.42 R61
2.12 R62
1.27 R63
1.27 R64
1.47 R65
2.09 R67
2.09 R68
1.00 NIH(mukit)
0.07 NIH(neo)

The levels of c-Kit protein and mRNA displayed a tight positive correlation (R = 0.91; $p<0.01$, Spearman Rank Correlation test (Zar, 1984)) (Figure 4.5.2). The lower levels in magnitude of surface protein compared to mRNA expression is most likely due to SLF-induced surface c-Kit downmodulation and/or interference of antibody binding to SLF occupied c-Kit receptors. Subsequently, c-*kit* mRNA expression was used as a more accurate measure for comparing relative c-*kit* expression levels between clones.

These clones were assayed for their ability to produce colonies in soft agar in the absence or presence of exogenous mSLF. In the presence of 100 ng/ml mSLF a significant correlation between the level of c-*kit* mRNA expression and the number of anchorage independent colonies was obtained (R = 0.53; $p<0.01$; Spearman Rank Correlation test) (Figure 4.5.3 A). The majority of clones expressing the lowest levels of c-*kit* mRNA (clones R31-R49; c-*kit*:*GAPDH* ratio relative to NIH(mu*kit*) pool ranging between 0.15-0.5) in general produced negligible levels of anchorage independent growth with a plating efficiency of 0.47 ± 0.61%. Clones expressing a 3.4 (on average) fold higher level of c-*kit* mRNA in the medium group (R33-R65; 0.9-1.47) produced colonies at a plating efficiency of 2.3 ± 0.66% and those expressing high levels (a further 1.7 (on average) fold increase) of c-*kit* (R55-R56; 1.50-2.52) produced colonies at a plating efficiency of 3.5 ± 1.0% in comparison to the 5.8 ± 0.14% plating efficiency of the NIH(mu*kit*) pool and 0.11 ± 0.0% by the NIH(*neo*) negative control. Figure 4.5.3.B. represents data obtained from a typical experiment. One of the clones NIH(mu*kit*).R43 reproducibly produced colonies at the same frequency in the presence and absence of mSLF. This clone did not express c-Kit protein or mRNA (refer to Figure 4.5.1B) and was therefore transformed by an event unrelated to c-*kit* expression. Interestingly, NIH(mu*kit*).R56, which expressed the highest level of c-*kit* mRNA in this study, reproducibly produced a negligible number of colonies (plating efficiency of 0.07%). It was possible that poor colony production of this clone reflected a lower proportion of the cells being in cycle. To investigate this all the clones were stained with propidium iodide on the same day as the colony assay (refer to section 2.3.3 for method) and analysed by flow cytometry. No differences in cell cycle parameters were observed (data not shown). In the absence of added mSLF only two clones displayed significant anchorage independent growth.

Figure 4.5.2: Comparison of c-Kit protein and mRNA levels in the NIH(mu*kit*) clones. Cell surface protein expression was determined by indirect immunofluorescence and flow cytometry as described previously in Figure 4.2.2, and results are expressed as mfi units. c-*kit* mRNA expression was determined by northern blot hybridisation as described in Figure 4.4.1 and the results expressed as the c-*kit/GAPDH* signal ratio relative to the NIH(mu*kit*) pool. ($R = 0.91$; $p<0.01$; Spearman Rank Correlation test). x represents NIH(mu*kit*) pool.

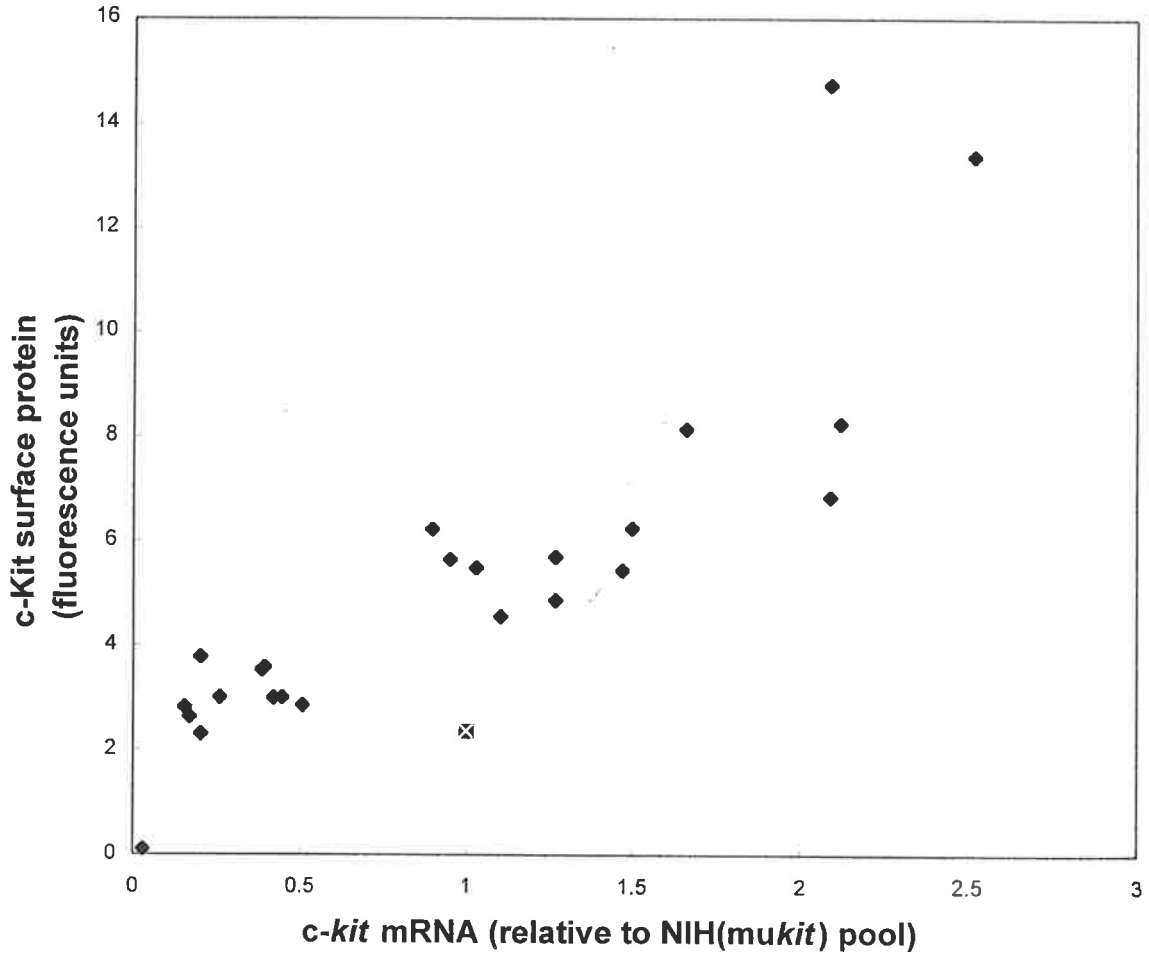

c-Kit surface protein
(fluorescence units)
16
14
12
10
8
6
4
2
0
0
0.5
1
1.5
2
2.5
3
c-*kit* mRNA (relative to NIH(mu*kit*) pool)

Figure 4.5.3 A: Comparison between c-*kit* mRNA expression levels and anchorage independence in the presence of mSLF. c-*kit* mRNA expression levels were determined as described in Figure 4.4.1 and the results expressed as the c-*kit/GAPDH* signal ratio relative to the NIH(mu*kit*) pool. Anchorage independence is expressed as the number of colonies produced by day 14 in the presence of 100 ng/ml of mSLF. x represents NIH(mu*kit*) pool ($R = 0.53$; $p<0.01$; Spearman Rank Correlation test).

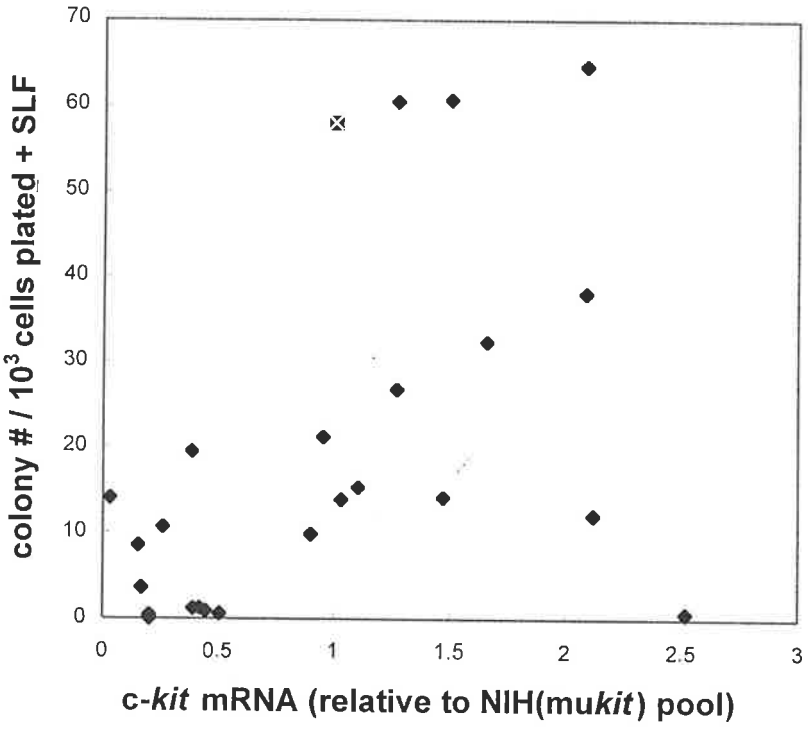
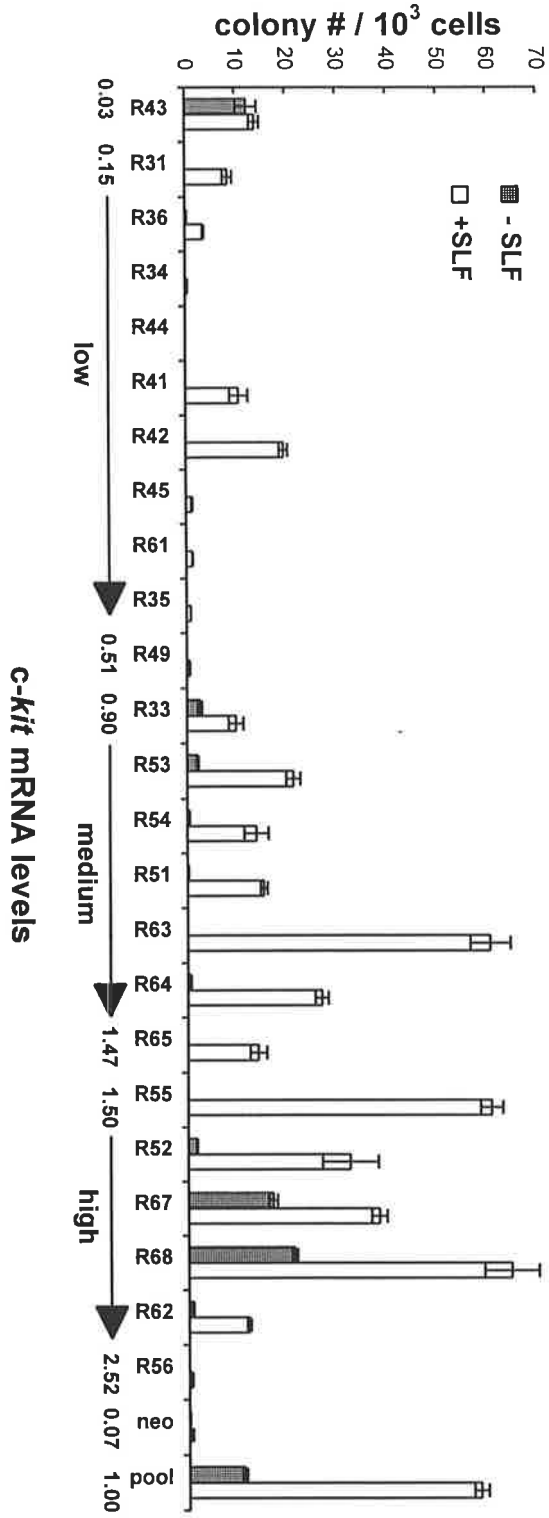

70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
2.5
3
colony # / 10^3 cells plated + SLF
c-kit mRNA (relative to NIH(mukit) pool)

Figure 4.5.3 B: Anchorage independent growth of the NIH(mu*kit*) clones (R31-R56) in soft agar. 10^4 cells were plated in soft agar in the absence or presence of 100 ng/ml mSLF were indicated. Colonies were scored on day 14. Clones are shown in order of increasing c-*kit* mRNA levels relative to the NIH(mu*kit*) pool determined as described in Figure 4.5.1 B. Colony numbers represented as the mean ± S.E.M. of quadruplicate replicates per 10^3 cells plated.


colony # / 10^3 cells
0
10
20
30
40
50
60
70
- SLF
+SLF
0.03
R43
0.15
R31
R36
R34
R44
R41
R42
R45
R61
R35
0.51
R49
0.90
R33
R53
R54
R51
R63
R64
1.47
R65
1.50
R55
R52
R67
R68
R62
2.52
R56
0.07
neo
1.00
pool
low
medium
high
c-kit mRNA levels

These clones, NIH(mu*kit*).R67 and R68, contained identical levels of c-*kit* mRNA and fell within the high c-*kit* expression group in this study, however other clones within this group did not produce colonies in the absence of exogenous mSLF. Due to the low frequency of colony production in the absence of mSLF by the majority of the clones, the cells were plated at a 5-fold higher density to increase the probability of colony production. Apart from NIH(mu*kit*).R67 and R68, no clones were able to produce colonies above the NIH(*neo*) background colony number when plated at a higher density, and as a consequence no significant correlation was obtained between anchorage independence and c-*kit* levels in the absence of exogenous mSLF ($R = 0.30$; $p > 0.05$) (Figure 4.5.4).

4.6 *Analysis of levels of SLF mRNA produced by the NIH(mukit) sorted and plucked clones*

Another factor apart from the level of c-*kit* expression which may contribute to anchorage independence of these clones, was the amount of SLF produced by the individual clones. The NIH(mu*kit*) clones described in section 4.4 and 4.5 expressed a major *SLF* mRNA transcript of approximately 6 - 6.5 kb (Huang *et al.*, 1990). Data for some of the clones analysed are shown in Figure 4.6.1. No significant correlation was seen between the levels of *SLF* mRNA and colony production in the absence or presence of added mSLF ($R = 0.22$, and $R = 0.34$; $p>0.05$, respectively) (Figure 4.6.2A, B). Interestingly, NIH(mu*kit*).R56 which expressed the highest level of c-*kit* also had the highest level of *SLF* mRNA (2 fold higher than the NIH(mu*kit*) pool) (refer to Figure 4.6.1) yet produced a negligible number of colonies. The cells from the plucked colonies NIH(mu*kit*).A-E were also analysed for their level of *SLF* mRNA production. All expressed lower levels of *SLF* than the NIH(mu*kit*) pool (Figure 4.6.1).

4.7 *Spontaneous transformants*

Balb/c *nude* mice were injected subcutaneously with 10^5 or 10^6 NIH(mu*kit*) pool and NIH(*neo*) cells to assess their ability to induce tumours *in vivo*. Initial experiments revealed that not only the NIH(mu*kit*) infectants but also the NIH(*neo*) control cells were able to produce tumours. Considering that the concentration of cells was too high, the experiment

Figure 4.5.4: Comparison between c-*kit* mRNA levels and anchorage independent growth of NIH(mu*kit*) clones in the absence of added mSLF. c-*kit* mRNA levels were determined by northern blot hybridisation and results expressed as the c-*kit/GAPDH* signal ratio relative to the NIH(mu*kit*) pool as described in Figure 4.4.1. Anchorage independence expressed as the number of colonies produced by day 14 in the absence of mSLF. ($R = 0.30$; $p>0.05$; Spearman Rank Correlation test). Solid square represents NIH(*neo*) control cells and x represents NIH(mu*kit*) pool.

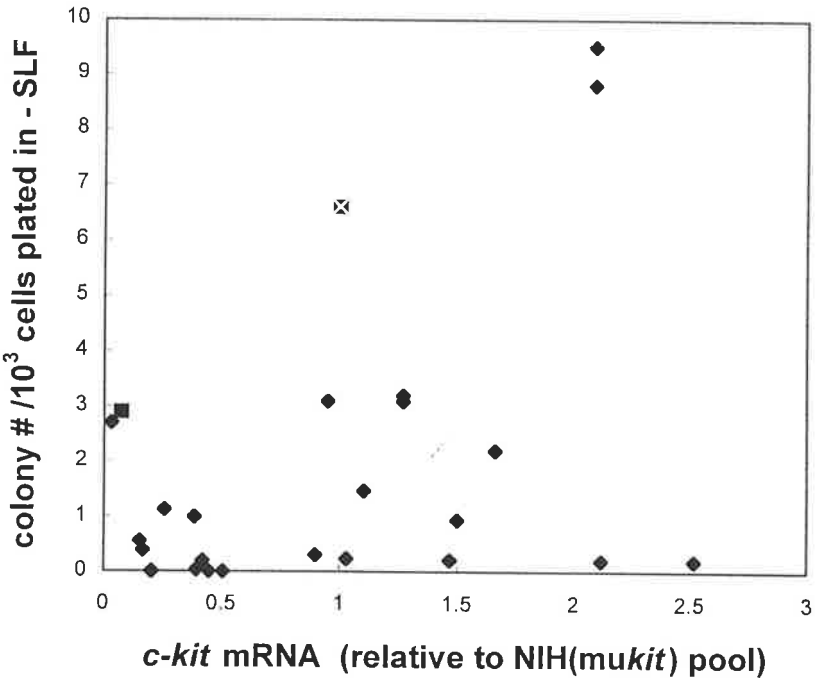

colony # /10^3 cells plated in - SLF
10
9
8
7
6
5
4
3
2
1
0
0
0.5
1
1.5
2
2.5
3
c-kit mRNA (relative to NIH(mukit) pool)

Figure 4.6.1: *SLF* mRNA expression in a selection of the NIH(mu*kit*) clones. 2μg of poly A^+ selected mRNA from NIH(mu*kit*) selected clones and NIH(*neo*) were analysed by agarose gel electrophoresis and northern blot hybridisation. Hybridisation was performed using a full length murine *SLF* probe and a 780 bp human *GAPDH* fragment labelled with ^{32}P. Hybridisation signals were visualised and quantitated using a Molecular Dynamics PhosphorImager as described in section 2.7.5. *GAPDH* signals were used as a control for comparing sample loading. *SLF* mRNA levels were represented as *SLF/GAPDH* signal ratios relative to the NIH(mu*kit*) pool.

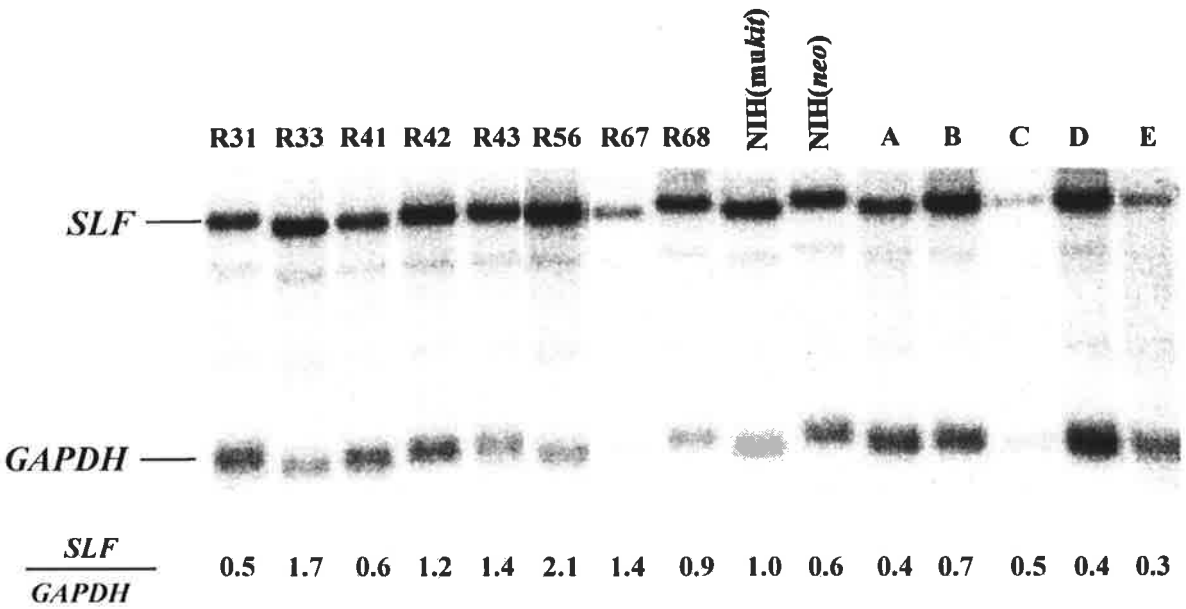
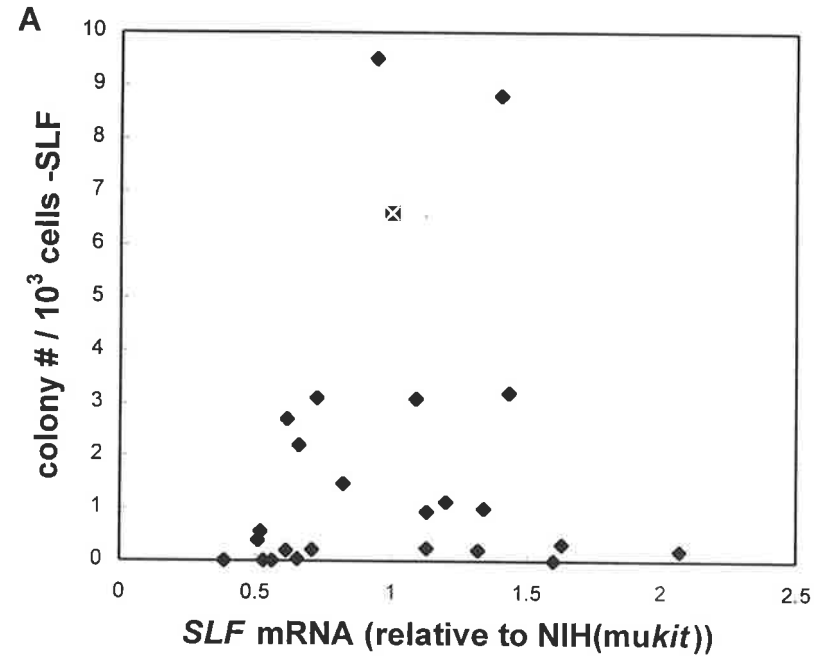
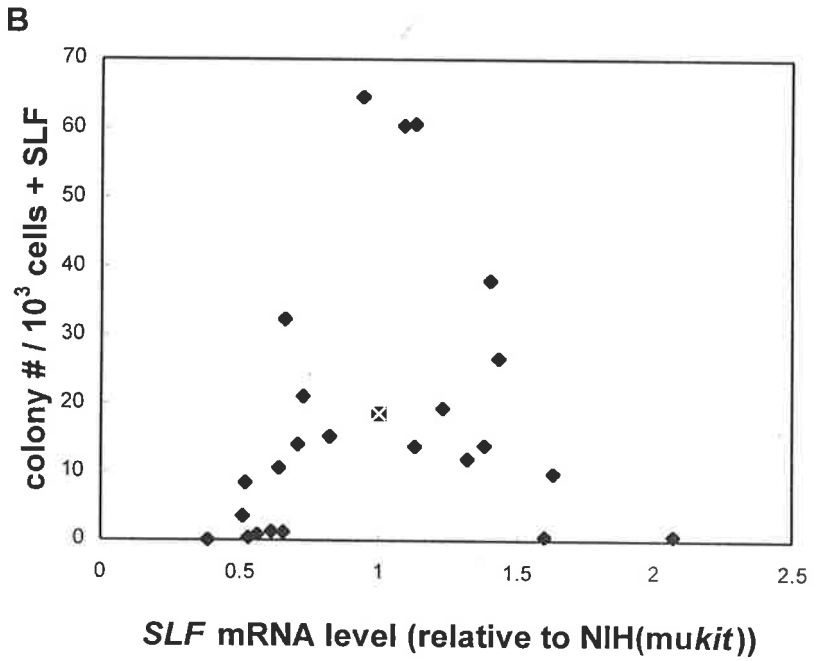

R31 R33 R41 R42 R43 R56 R67 R68 NIH(mu*kit*) NIH(*neo*) A B C D E
SLF
GAPDH
SLF / *GAPDH*
0.5 1.7 0.6 1.2 1.4 2.1 1.4 0.9 1.0 0.6 0.4 0.7 0.5 0.4 0.3

Figure 4.6.2: Comparison between *SLF* mRNA expression levels and anchorage independence in the absence of exogenous mSLF (A) and in the presence of 100 ng/ml mSLF (B). Anchorage independence is represented as the number of colonies scored on day 14 per 10^3 cells plated. *SLF* mRNA levels were determined as described in Figure 4.6.1. x represents NIH(mu*kit*) pool. (A. R = 0.22 and B. R = 0.33; $p>0.05$; Spearman Rank Correlation test)


A
colony # / 10^3 cells -SLF
0
1
2
3
4
5
6
7
8
9
10
0
0.5
1
1.5
2
2.5
SLF mRNA (relative to NIH(mukit))

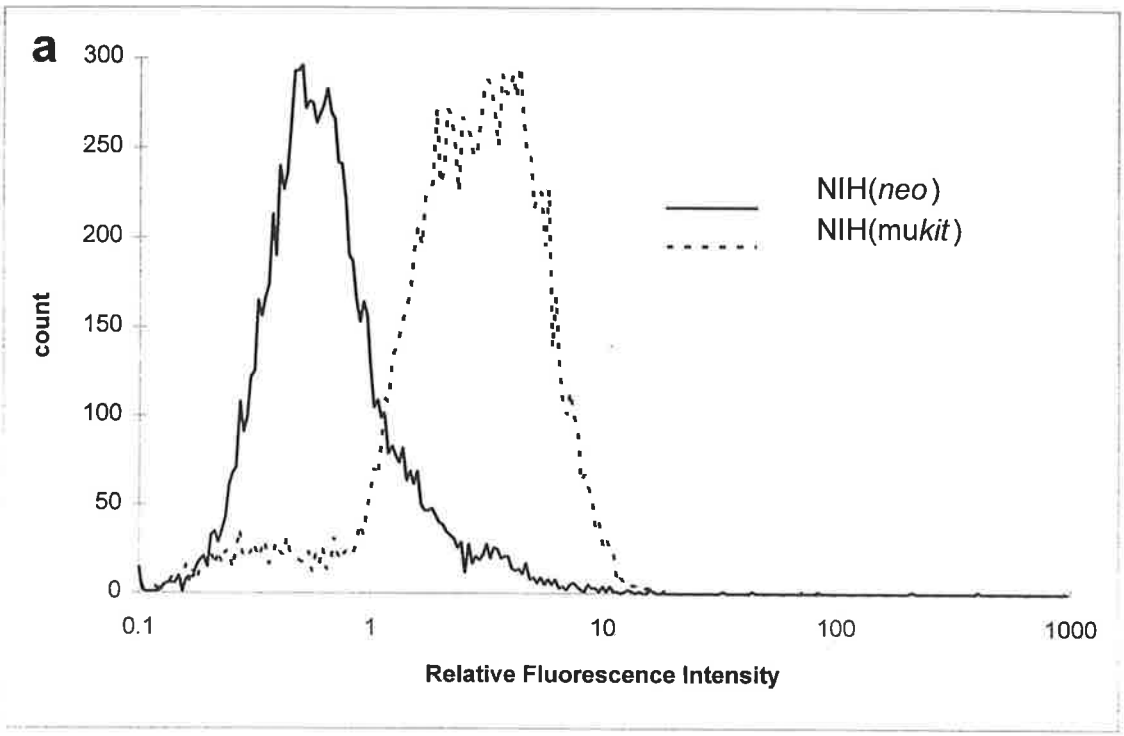

B
colony # / 10^3 cells + SLF
0
10
20
30
40
50
60
70
0
0.5
1
1.5
2
2.5
SLF mRNA level (relative to NIH(mukit))

was repeated using 10^4 cells/mouse. 4/6 mice receiving NIH(mu*kit*) cells developed tumours with a latency period of approximately 46 days while 2/6 mice injected with control cells also developed tumours. It became evident that the NIH/3T3 cells were partially transformed, the problem only becoming evident upon injecting cells into *nude* mice.

4.8 *Infection of a new early passage NIH/3T3 cell line*

Since no conclusions could be drawn in regards to the ability of NIH(mu*kit*) to induce tumours *in vivo* within this NIH/3T3 background it was decided to infect an early passage NIH/3T3 line. These cells, specifically recommended by ATCC for use in transformation and tumourigenicity assays (referred to as epNIH/3T3 from here on to distinguish them from the NIH/3T3 cells previously used), were obtained from Elizabeth MacMillian (Hanson Centre for Cancer Research, Adelaide, Australia). epNIH/3T3 cells were infected with viral supernatant from the X36 ψ2 clone and also from the Zen*neo* expressing ψ2 pool as described previously for the NIH(mu*kit*) and NIH(*neo*) cells, respectively. The resultant infectants epNIH(mu*kit*) expressed comparable levels of c-Kit surface protein to the NIH(mu*kit*) pool of infectants as portrayed in Figure 4.8.1. These new infectants were initially tested for their ability to grow in soft agar; the epNIH(mu*kit*) pool in the presence of 100 ng/ml mSLF gave an average plating efficiency of 4% compared to 12% with NIH(mu*kit*) cells and 0.3% compared to 2.5% in the absence of added mSLF, respectively (refer to Table 4.8.1). Since the levels of c-Kit in these two infected lines were comparable, the differences in plating efficiency can be attributed to the different NIH/3T3 background. Even in this early passage NIH/3T3 cell background colonies were also produced in the absence of added mSLF. This further verified that the colonies produced in the initial NIH/3T3 cells used in this chapter, were not produced as a secondary event associated with fact that these cells displayed signs of spontaneous transformation. To confirm the data obtained with the old NIH(mu*kit*) clones expressing increasing levels of c-*kit* described in section 4.5, pools of epNIH(mu*kit*) cells that represented low (epNIH(mu*kit*)-R2), medium (epNIH(mu*kit*)-R3) and high (epNIH(mukit)-R4) levels of c-Kit expression in relation to the original epNIH(mu*kit*) pool were selected by fluorescence-activated cell sorting (Figure 4.8.2).

Figure 4.8.1: Expression of surface c-Kit protein on the early passage NIH/3T3 cell infectants. Fluorescence histograms illustrating the surface expression of c-Kit detected by indirect immunofluorescence as described in Figure 4.2.2 on both pZen(*neo*) (——) and pZen(mu*kit*) (·····) fibroblast infectants. (a) NIH/3T3, (b) early passage (ep) NIH/3T3 cells.


a
300
250
200
150
100
50
0
count
0.1
1
10
100
1000
Relative Fluorescence Intensity
NIH(neo)
NIH(mukit)

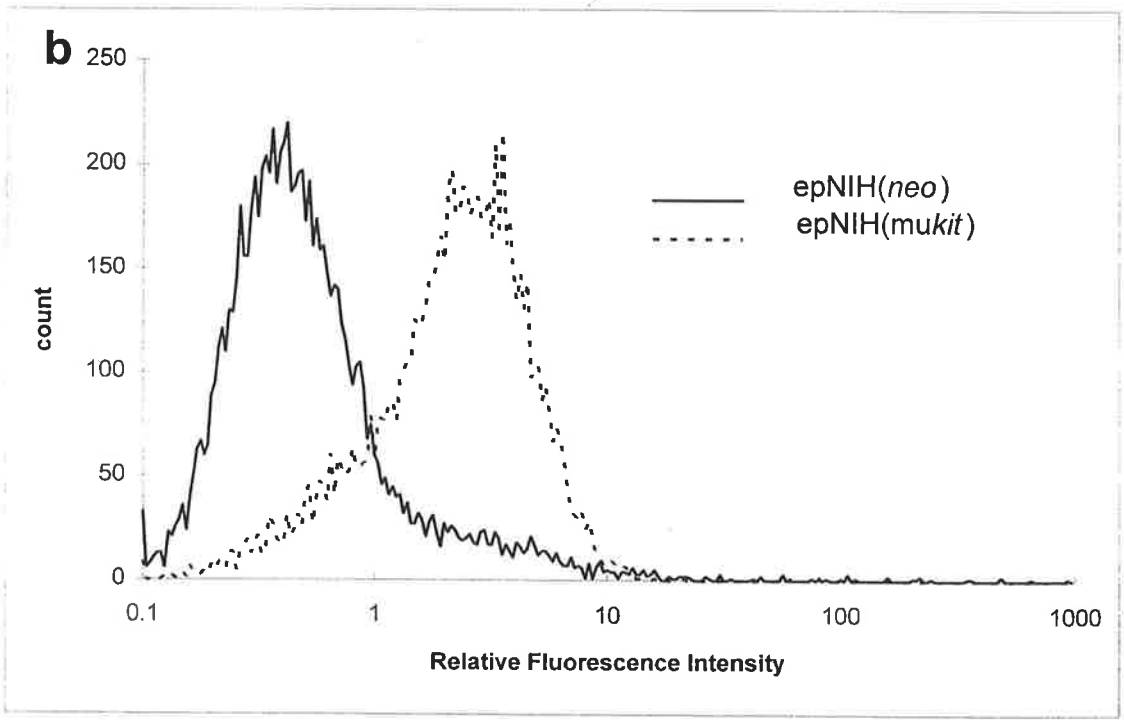
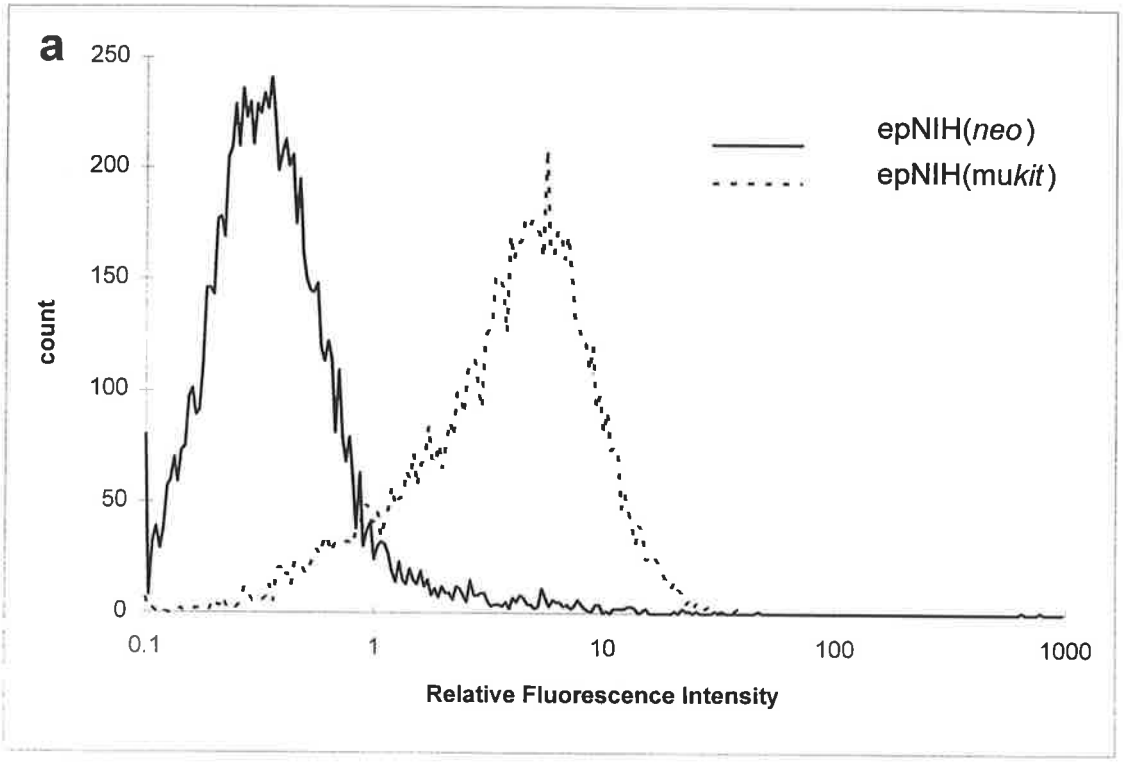

b
250
200
150
100
50
0
count
0.1
1
10
100
1000
Relative Fluorescence Intensity
epNIH(neo)
epNIH(mukit)

Figure 4.8.2: Expression of surface c-Kit protein on cells derived from sorted populations of the epNIH(mu*kit*) cells. Fluorescence histograms illustrating the surface expression of c-Kit detected by indirect immunofluorescence as described in Figure 4.2.2. (a) epNIH(mu*kit*) and epNIH(*neo*), (b) epNIH(mu*kit*)-R2, R3, R4.


a
250
200
150
100
50
0
count
0.1
1
10
100
1000
Relative Fluorescence Intensity
epNIH(neo)
epNIH(mukit)

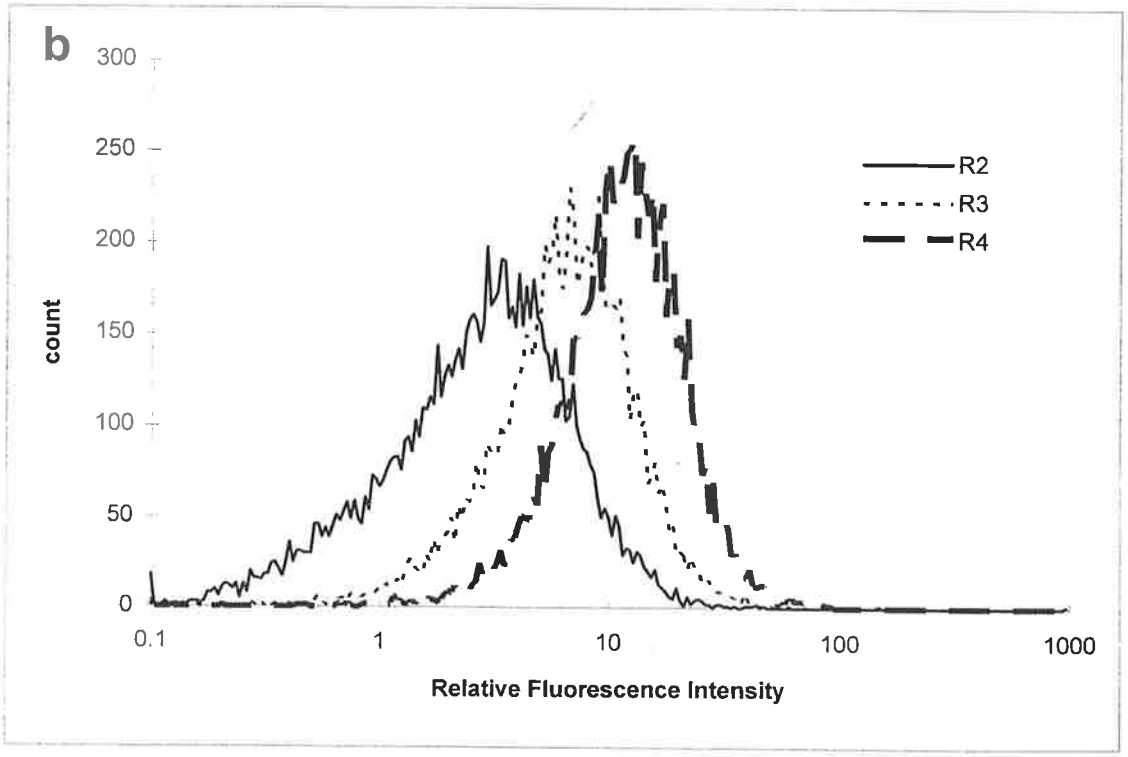

b
300
250
200
150
100
50
0
count
0.1
1
10
100
1000
Relative Fluorescence Intensity
R2
R3
R4

Table 4.8.1: Anchorage independence of the NIH/3T3 cells expressing murine c-Kit.

NIH/3T3 infectants	colonies / 10^3 cells	
	- mSLF	+ mSLF
epNIH(*neo*)	0 ± 0	0 ± 0
epNIH(mu*kit*)	2.75 ± 0.44	39.38 ± 1.45
epNIH(mu*kit*)-R2	7.40 ± 1.43	53.65 ± 5.45(3)
epNIH(mu*kit*)-R3	5.65 ± 0.24	132.00 ± 5.15
epNIH(mu*kit*)-R4	17.15 ± 2.68	221.38 ± 20.10
NIH(*neo*)	0 ± 0	0 ± 0
NIH(mu*kit*)	24.75 ± 5.00	122.67 ± 4.9(3)

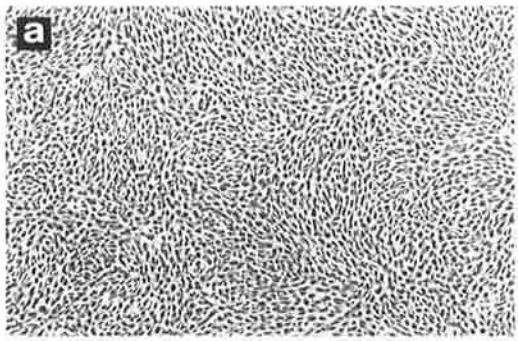
2 x 10^3 cells were plated in 0.33% semi-solid agar in the absence or presence of 100 ng/ml mSLF. Colonies consisting of >50 cells were scored microscopically after 14 days. Colony numbers represented as the mean ± S.E.M of quadruplicate replicates unless otherwise indicated in parenthesis.

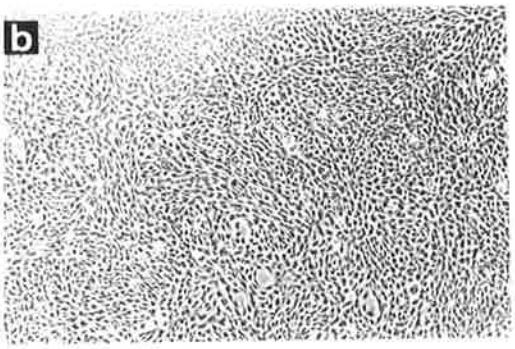
The level of c-Kit expressed by these pools affected their morphology when cultured in the presence of 100 ng/ml of mSLF. In the absence of added mSLF the cells showed little alteration compared to the epNIH(*neo*) infectants (although epNIH(mu*kit*)-R2 and -R4 monolayers appeared more dense (Figure 4.8.3 d, f)) and all populations reached confluence within 3 days (Figure 4.8.3 a, b, d, f). In the presence of mSLF morphological changes were evident and the cells had not become confluent by day 3. In the presence of mSLF all populations except the NIH(*neo*) cells appeared spindly and grew in a disorganised manner with the appearance of refractile cells. epNIH(mu*kit*) and epNIH(mu*kit*)-R2 displayed similar morphologies (Figure 4.8.3 c, e). epNIH(mu*kit*)-R4, however produced a striking and reproducible morphological change, in which the cells grew in distinct foci and the cells were less adherent to the substratum (Figure 4.8.3. g). As a consequence of this loss of contact dependence epNIH(mu*kit*)-R4 did not reach confluence throughout the culture period (12 days). epNIH(mu*kit*) and epNIH(mu*kit*)-R2 also showed signs of loss of contact dependence but to a lesser extent.

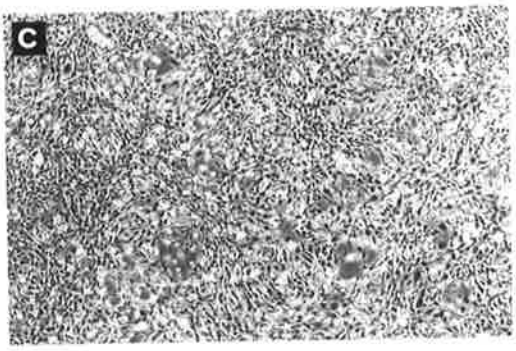
These cells also demonstrated the correlation previously described in Figure 4.5.3 A, B in which anchorage independence increased with c-*kit* expression levels. epNIH(mu*kit*)-R4 displayed approximately 2 and 4 fold higher levels of anchorage independent growth than those expressing the lowest level of c-*kit,* epNIH(mu*kit*)-R2, in the absence or presence of 100 ng/ml SLF, respectively (Table 4.8.1).

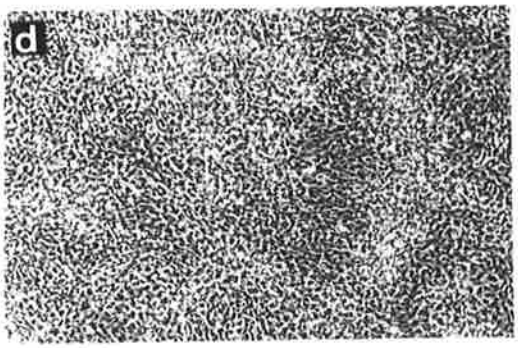
The levels of c-*kit* mRNA of these selected pools are represented in Table 4.8.2. epNIH(mu*kit*)-R4 expressed 2.3 fold higher levels of c-*kit* mRNA than the lower expressing epNIH(mu*kit*)-R2 cell population. Using the level of c-*kit* mRNA in NIH(mu*kit*) as a reference, it was shown that epNIH(mu*kit*)-R2, R3 expressed low-medium levels, and -R4 expressed high levels of c-*kit* in comparison to the clones described in section 4.5 (refer to Figure 4.5.3 B and Table 4.8.2). The original epNIH(mu*kit*) pool from which these latter populations were derived expressed equivalent levels of c-*kit* mRNA to that expressed by the NIH(mu*kit*) cells as expected from the comparisons previously made at the surface protein level (Figure 4.8.1). By comparison, the level of c-*kit* mRNA in the NIH(mu*kit*) pools (ie the

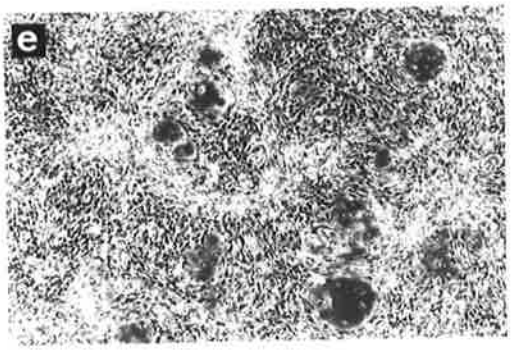
Figure 4.8.3: Morphology of epNIH(mu*kit*) infectants expressing various levels of c-Kit. 5 x 10^4 cells were cultured in the absence or presence of 100 ng/ml of mSLF. (a) epNIH(*neo*) + mSLF, (b) epNIH(mu*kit*) - mSLF, (c) epNIH(mu*kit*) + mSLF, (d) epNIH(mu*kit*).R2 - mSLF, (e) epNIH(mu*kit*).R2 + SLF, (f) epNIH(mu*kit*).R4 - SLF, (g) epNIH(mu*kit*).R4 + mSLF. Cultures were fed twice weekly for a period of 10 days. Photographs of representative regions taken on day 6 under phase contrast optics at 4x magnification.

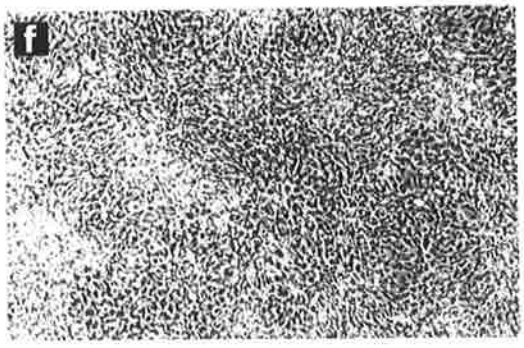

a


b


c


d


e


f

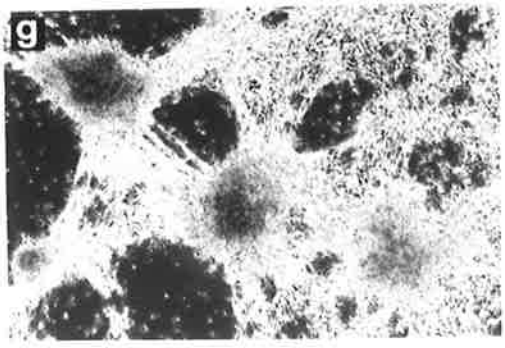

g

Table 4.8.2: Expression of c-*kit* mRNA in a selection of cell lines.

cell line	c-*kit* mRNA*
epNIH(*neo*)	0.01
epNIH(mu*kit*)	1.09
epNIH(mu*kit*)-R2	0.70
epNIH(mu*kit*)-R3	0.95
epNIH(mu*kit*)-R4	1.63
NIH(*neo*)	0.01
NIH(mu*kit*)	1.00
P815	0.50
FDC-P1(SLF)	0.09

*c-*kit* mRNA levels determined by northern blot analysis and the hybridisation signal quantified using Molecular Dynamics PhosphorImager. Signals represented as c-*kit/GAPDH* ratio relative to NIH(mu*kit*) cells allowing comparisons to be made with data represented in Figure 4.5.1 B.

original epNIH(mu*kit*) and the old NIH(mu*kit*) cells) were 2 fold higher than that expressed in the murine mastocytoma cell line, P815 (Table 4.8.2).

4.9 *Tumourigenicity in nude mice*

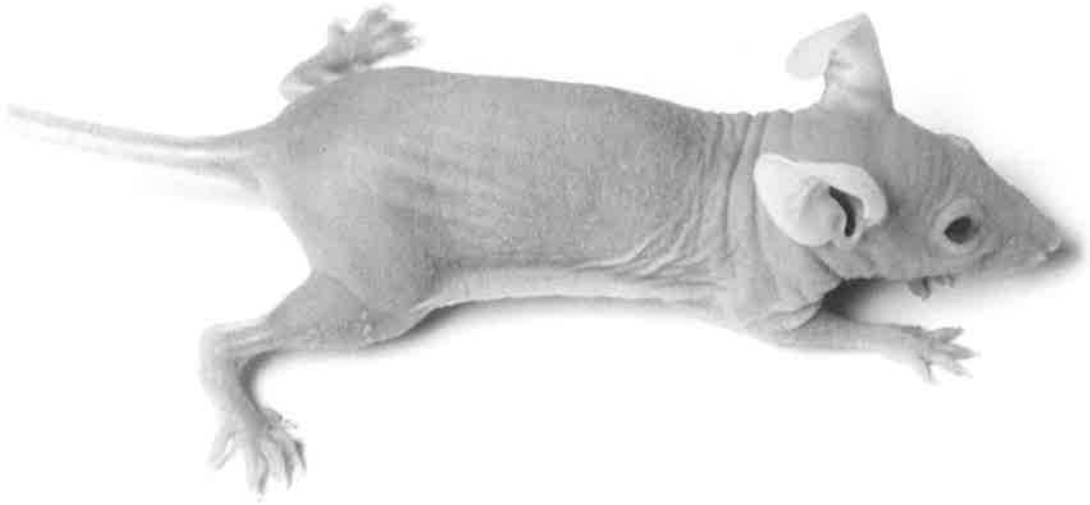
The new early passage c-*kit* infectants, epNIH(mu*kit*) were assayed for their ability to produce tumours in *nude* mice. Initial experiments demonstrated that up to 10^7 cells could be injected into these mice with tumours developing from injection of epNIH(mu*kit*) cells but not the epNIH(*neo*) cells (Figure 4.9.1). Subsequent experiments including the subcutaneous injection of epNIH(mu*kit*)-R2 and -R4 demonstrated that epNIH(mu*kit*)-R4 cells expressing the highest levels of c-Kit produced slightly more rapid tumours in *nude* mice than epNIH(mu*kit*)-R2 cells or the original epNIH(mu*kit*) cells (Table 4.9.1.).

4.10 DISCUSSION

Transformation based on anchorage independent growth of NIH/3T3 cells induced by overexpression of a human chimeric c-Kit receptor (Lev *et al.*, 1990) and by the full length murine c-Kit receptor (Alexander *et al.*, 1991) has previously been reported, but whether these cells displayed the full spectrum of phenotypic changes characteristic of transformation was not demonstrated. Neither the level of receptor expression nor the absolute requirement for ligand for transformation to occur were investigated. In this study, we demonstrate that high levels of the normal murine c-Kit receptor in NIH(mu*kit*) and epNIH(mu*kit*) pools of infectants resulted in a fully transformed phenotype. These included morphological changes as well as the ability to grow in low levels of serum, produce foci, grow in an anchorage independent manner in soft agar and produce tumours in *nude* mice.

To investigate the effect of c-Kit receptor levels on NIH/3T3 transformation a series of clones, NIH(mu*kit*).R31-R56, expressing increasing levels of c-*kit* mRNA and protein were analysed for their ability to induce anchorage independent growth in the presence of mSLF. Clones grouped as expressing low levels of c-*kit* (R31-R49) were generally unable to produce colonies (plating efficiency of ~ 0.47%) in comparison to clones expressing approximately 3.4 fold (on average) higher levels in the medium group (R33-R65; plating efficiency of ~ 2.3%)

Figure 4.9.1: Tumour formation induced by murine c-*kit*. 8 x 10^6 cells were injected subcutaneously into Balb/c *nude* mice. Mice were monitored twice weekly and tumours measured. Photograph represents mice injected with epNIH(*neo*) control cells (top photograph) and epNIH(mu*kit*) cells (bottom photograph).



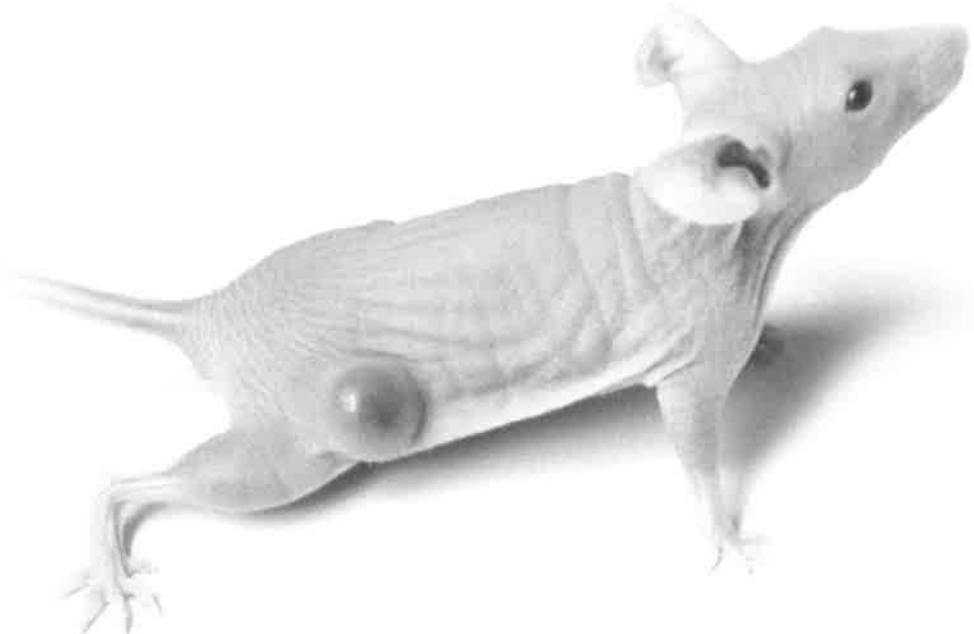


Table 4.9.1: Tumourigenicity produced by epNIH(mu*kit*) cells expressing various levels of c-Kit.

epNIH/3T3 infectants	latency period (days) for 50% tumour incidence*
epNIH(mu*kit*)	48 ± 2.2
epNIH(*neo*)	>71
epNIH(mu*kit*)-R2	39 ± 0.0
epNIH(mu*kit*)-R4	32 ± 1.3

*Groups of 3 mice were injected subcutaneously with 8 x 10^6 cells at two sites (left and right hind flanks). Mice were monitored twice weekly and tumours > 2mm in size were measured on each visit. Latency period estimated as the mean time taken for tumours > 2 mm to form ± S.E.M. at 3 out of the 6 injection sites. At the termination of the experiment (day 71) no tumours were induced by the epNIH(*neo*) control cells and the number of tumours formed / 6 injection sites for epNIH(mu*kit*) and epNIH(mu*kit*)-R4 cells were 5/6 and for epNIH(mu*kit*)-R2 cells 6/6.

and those expressing a further 1.7 fold (on average) increase (R33-R65) producing the greatest plating efficiency of ~ 3.5%. A positive correlation existed between the level of c-*kit* mRNA expressed by these clones and anchorage independent growth in the presence of mSLF ($R = 0.53$; $p<0.01$). These results were consistent with the transformation obtained by the epNIH(mu*kit*)-R2, R3 and R4 pools of cells expressing increasing levels of c-*kit*. NIH(mu*kit*)-R4 cells which had the highest levels of c-*kit* displayed more dramatic changes in morphology in the presence of exogenous mSLF and an increase in anchorage independence than epNIH(mu*kit*)-R2 which expressed 2.3 fold lower levels of c-*kit* mRNA. Injection of epNIH(mu*kit*)-R2 and -R4 cells into *nude* mice were also consistent with the *in vitro* observations. The production of tumours may also be factor-dependent as a result of the stimulation of c-Kit on the epNIH(mu*kit*) cells injected with SLF produced by the mice.

Interestingly NIH(mu*kit*)-R56 and -R62, which expressed the highest levels of c-*kit*, produced negligible and low numbers of colonies, respectively, even in the presence of recombinant mSLF. Whether this is related to c-*kit* expression would have required the analysis of more clones expressing equivalent c-*kit* levels. Attempts to isolate additional clones expressing c-*kit* at equivalent or higher levels to these clones were unsuccessful suggesting that such cells were rare within this population and that extremely high levels of c-Kit may possibly incur a growth disadvantage. Interestingly NIH(mu*kit*)-R56 also had the highest levels of *SLF* mRNA. In general no correlation was seen between *SLF* mRNA expression and anchorage independent growth, however the combination of elevated c-*kit* and *SLF* in NIH(mu*kit*)-R56 may result in the inhibition of anchorage independent growth. This may be analogous to the inhibition of proliferation demonstrated in the carcinoma cell line, A431, and transfected cell lines expressing high levels of the EGF or HER-2/*neu* receptors when exposed to ligand. In contrast, a positive proliferative response was produced when these cells were stimulated with lower ligand concentrations, or by cells expressing lower receptor levels (Kawamoto *et al.*, 1984; Lupu *et al.*, 1990; Riedel *et al.*, 1987). Although the limited cell cycle analysis of c-*kit* expressing clones did not suggest that NIH(mu*kit*)-R56 and -R62 cells behaved any differently in the absence of mSLF to the other clones it is possible

that the elevated levels of c-*kit* and/or *SLF* may effect the specific events necessary for anchorage independent growth.

In order to compare the levels of c-*kit* expressed by these clones with physiological levels, comparisons were made between NIH(mu*kit*) and epNIH(mu*kit*) infectants with the murine mastocytoma cell line, P815. It has been demonstrated that the levels of cell surface c-Kit protein expressed by P815 cells are comparable to those found on normal cultured mast cells (Tsujimura *et al.*, 1994). Human mast cells express the highest levels of c-Kit found *in vivo* (Mayrhofer *et al.*, 1987; L.K. Ashman, unpublished data). The levels of c-*kit* mRNA in the pool NIH/3T3 infectants exceeded those expressed in P815 by 2 fold; the mRNA levels expressed by P815 are equivalent to those clones expressing low-medium levels (Table 4.8.2 and Figure 4.5.3 B). Thus transformation of NIH/3T3 cells appeared to require expression of c-*kit* at greater than physiological levels.

Of particular interest in this study was whether c-Kit receptor levels could contribute to the induction of factor-independent transformation in a similar way to that proposed for the HER-2/*neu* receptor. Overexpression of the HER-2/*neu* receptor has been suggested to drive spontaneous dimerization in the absence of ligand (Brandt-Rauf *et al.*, 1990; Samanta *et al.*, 1994). The ability of the pool NIH(mu*kit*) and epNIH(mu*kit*) infectants to produce anchorage independent colonies in the absence of exogenous mSLF suggested that this may occur with the c-Kit receptor in some of the cells within these pools. The increase in plating frequency demonstrated by epNIH(mu*kit*)-R2, -R3 and -R4 cells progressively expressing increasing c-Kit levels also suggested that receptor levels influenced the degree of anchorage independence in the absence of exogenous mSLF. Whether these colonies were being produced in a factor-independent manner could not be resolved in the NIH/3T3 background since it was most likely that c-Kit stimulation was occurring by endogenous SLF, therefore completing an autocrine loop. Unfortunately, introduction of c-*kit* in *Sl/Sl*-3T3 cells which are devoid of endogenous SLF (refer to Chapter 3) could not induce transformation even in the presence of added ligand so that this question could not be easily addressed. However analysis of the clones NIH(mu*kit*).R31-R56 demonstrated that only 2 of the 24 clones analysed, NIH(mu*kit*)-R67 and -R68, could grow in an anchorage independent manner in the presence

of endogenous SLF alone. These cells had been assigned to the high c-*kit* expressing group. It has been reported that high levels of the EGFR are required for the induction of anchorage independence of NIH/3T3 cells stimulated by autocrine TGF-α alone (Di Marco *et al.*, 1989), and that cells expressing lower levels of the EGFR did not show signs of transformation (Finzi *et al.*, 1987). In the cells exhibiting transformation it appears that a threshold level of EGFR expression is required to induce this phenotype. The increase in receptor levels may possibly facilitate more efficient dimerization culminating in more enhanced EGFR signal transduction leading to transformation. It is possible that similar interactions were occurring in this study for NIH(mu*kit*)-R67 and R68, although this was not a general phenomenon of other clones in this high c-Kit expression group which were unable to grow in an anchorage independent manner in the presence of endogenous SLF alone. It should be noted that anchorage independent growth of NIH(mu*kit*)-R67 and R68 in the absence of added mSLF could not be attributed to abnormally high levels of *SLF* mRNA expression. Thus analysis of the clones expressing increasing receptor densities did not fully explain why anchorage independent growth could be obtained by the pool NIH(mu*kit*) and epNIH(mu*kit*) infectants in the absence of exogenous mSLF.

Further investigation into whether the colonies produced by NIH(mu*kit*) and epNIH(mu*kit*) pools in the absence of exogenous mSLF were a result of autocrine stimulation demonstrated that these colonies were resistant to the antagonistic α-c-Kit mAb, ACK2, and to the partially neutralising α-SLF antibody. While at first sight this suggested that autocrine SLF was not important it is possible that the interaction between endogenously produced SLF and the c-Kit receptor was not occurring at the cell surface but that an intracellular interaction was taking place. This has been demonstrated in cells co-expressing the PDGFR and the v-*sis*/c-*sis* gene product (Huang & Huang, 1988; Keating & Williams, 1988), and has also been suggested for the interactions of co-expressed CSF-1R/CSF (Sherr, 1988) and GM-CSFR/GM-CSF (Lang *et al.*, 1985), where antisera were unable to inhibit transformation. Alternatively, since SLF can interact with c-Kit at the cell surface, membrane-associated or secreted SLF may preferentially bind to the receptor over antibody due to local concentration effects. Attempts to inhibit SLF autocrine stimulation of human c-Kit in a number of

carcinoma cell lines with an antagonistic anti-c-Kit mAb, SR-1 have also been unsuccessful (Turner *et al.*, 1992). Thus, the lack of inhibition of colony formation in the absence of added mSLF by blocking antibodies has not excluded a requirement for autocrine SLF. Attempts to try and block the possible intracellular interactions between c-Kit and SLF are currently being investigated through the use of anti-sense *SLF* cDNA constructs.

It was possible that the colonies resistant to antisera inhibition in the absence of added mSLF were arising due to events independent of c-Kit in a similar manner to that demonstrated by the spontaneous transformant NIH(mu*kit*).R42 cells. Clones derived from colonies produced in the absence of added mSLF, NIH(mu*kit*).A-E, were therefore directly analysed for their expression of c-Kit and responsiveness to mSLF. These clones however were demonstrated to possess functional c-Kit receptors displaying a tight range of c-Kit surface protein comparable to the medium expressing clones NIH(mu*kit*).R33-R65. NIH(mu*kit*).A-E cells produced relatively high plating efficiencies especially in the absence of mSLF (upon replating) in contrast to the R33-R65 medium c-*kit* expressing clones. Neither the levels of c-*kit* nor *SLF* mRNA expressed in these cells could explain why NIH(mu*kit*).A-E displayed anchorage independent growth in the absence of added mSLF suggesting that another event(s) may be involved. These could be due to alterations to the receptor or clonal variations in the downstream signalling pathways. Future analysis on the degree of tyrosine phosphorylation associated with these receptors may explain the enhanced transformation seen. If abnormal phosphorylation is observed further analysis will be required to determine whether the high frequency of transformation in NIH(mu*kit*).A-E and also NIH(mu*kit*).R67 and R68 in the absence of added mSLF may be a result of spontaneous mutations introduced in c-*kit* during retroviral infection (not detectable by northern blot analysis). Expression of the genes encoding these potentially altered c-Kit receptors in a factor-dependent cell line would identify whether these receptors could abrogate the need for SLF. Mutations inflicting constitutive activation of several receptor tyrosine kinases resulting in ligand-independent transformation have been identified in this way (Pierce *et al.*, 1990; Kitayama *et al.*, 1995; Glover *et al.*, 1995).

It should also be noted that an alternate splice variant of c-*kit*, has been isolated. This variant differs from that used in this study by the insertion of four amino acids ($GNNK^+$) in the juxtamembrane of the extracellular domain (Reith *et al.,* 1991). The lack of these four amino acids ($GNNK^-$) in c-Kit has been associated with its ability to show low levels of constitutive phosphorylation in the absence of ligand in comparison to $GNNK^+$. It is possible that the $GNNK^-$ isoform used in this chapter may also contribute to the transformation ability of these cells in the absence and presence of mSLF. Analysis of the transforming potential of these two naturally occurring c-Kit isoforms will be addressed in Chapter 5.

CHAPTER 5

The transforming potential of the natural occurring isoforms of the human c-Kit receptor tyrosine kinase.

5.1 INTRODUCTION

In the previous chapter it was shown that an increase in murine c-*kit* expression correlated with enhanced factor-dependent transformation of NIH/3T3 cells. The sequence of the murine c-*kit* cDNA used in Chapter 4 corresponded to that published by Qui *et al.*, (1988). It was later reported that a second naturally occurring splice variant of c-*kit* existed in mice; this was generated by the use of an alternative 5' splice donor site at the exon/intron junction of exon 9 (Reith *et al.*, 1991; Hayashi *et al.*, 1991; and refer to Figure 5.3.1). These alternatively spliced c-*kit* mRNA transcripts differ in the in-frame deletion/insertion of 12 base pairs encoding the 4 amino acids, Gly-Asn-Asn-Lys (GNNK), located in the juxtamembrane region of the extracellular domain of the c-Kit receptor. The isoform lacking these 4 amino acids (GNNK-) displayed a low level of constitutive autophosphorylation in the absence of ligand and an association with phosphatidylinositol 3'-kinase (PI 3'-K) and phospholipase C-γ1 (PLC-γ1). Whereas these features were strictly ligand-dependent in the case of the c-Kit(GNNK+) isoform (Reith *et al.*, 1991).

c-Kit(GNNK-/+) isoforms are also expressed in humans, along with a second set of mRNA splice variants which differ in the in-frame insertion/deletion of 3 base pairs encoding a serine residue (S) at position 715. S+/- is located in the cytoplasmic domain lying within the interkinase region (Crosier *et al.*, 1993). In mice, the equivalent alternative splice variant does not exist due to lack of the alternative 3' splice acceptor site at the intron/exon junction of exon 15, which is found only in the human sequence (Vandenbark *et al.*, 1992; André *et al.*, 1992; and refer to Figure 5.3.1). Thus in mice all c-Kit is of the S- isoform. mRNAs encoding the c-Kit isoforms are co-expressed in normal bone marrow cells, normal melanocytes, several leukaemic cell lines, and acute myeloid leukaemic (AML) cells (Crosier *et al.*, 1993; Furitsu *et al.*, 1993; Piao *et al.*, 1994; Zhu *et al.*, 1994) with the c-Kit(GNNK-) isoform being more abundant than c-Kit(GNNK+) and c-Kit(S+) more frequent than c-Kit(S-). The function of these various isoforms remains to be elucidated.

Since the c-Kit(S+) isoform does not exist in mice, and since we also have better reagents, especially monoclonal antibodies (mAb), for the analysis of the human c-Kit receptor, the latter was examined in the studies described in this Chapter. The focus of these

experiments was on whether the various human c-Kit isoforms (GNNK+/-, S+/-) displayed any differences in their ability to transform NIH/3T3 cells. As in Chapter 4, the effect of c-Kit receptor levels was also addressed as a representative model of what may be occurring in acute myeloid leukaemia (AML), where high levels of c-Kit appeared to contribute to leukaemogenesis (Ashman *et al.*, 1988).

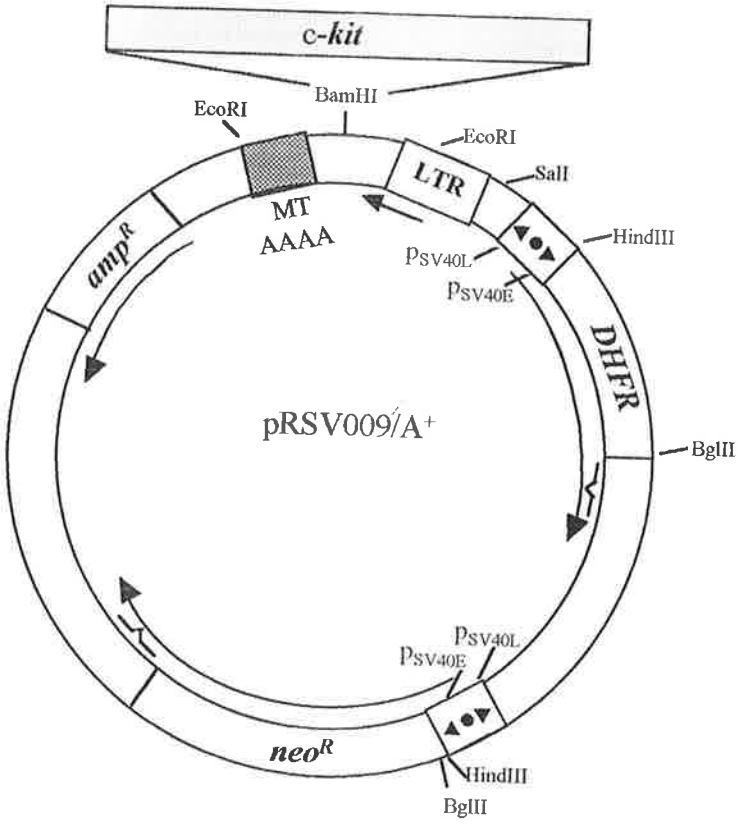
RESULTS

5.2 *Obtaining NIH/3T3 cells expressing increasing levels of c-Kit surface protein by methotrexate selection*

A different approach to that used in Chapter 4 for obtaining cells expressing increasing levels of c-Kit was employed in this study using the c-Kit(GNNK+S+) isoform (in accordance to the sequence published by Yarden *et al.*, 1987a). c-*kit(GNNK+S+)* cDNA harboured in pBluescript(SK) (pBS(SK)) (obtained from Dr. D. Williams, Immunex Corporation, Seattle, WA) was excised using Asp 718 - Not I, end-filled and cloned into the Bam HI site of the pRSV009/A+ eukaryotic expression vector (see methods described in section 2.4 for more detail), placing it under the control of the RSV-LTR. The resultant plasmid construct, designated pGC1.2 (see Figure 5.2.1), was introduced into NIH/3T3 cells by calcium phosphate mediated transfection (refer to section 2.8.2 for method). The expression vector contained the neomycin resistance gene (*neo*^{R}) allowing for selection of primary transfectants due to resistance to the antibiotic geneticin (G418). The vector also possessed the dihydrofolate reductase (*DHFR*) gene which would enable co-amplification of the linked c-*kit* gene when cells were placed under methotrexate (Mtx) selection. Isolation of a clone expressing c-Kit and subsequent culture in medium containing increasing concentrations of Mtx would enable progressive c-Kit amplification to be studied in an identical cell background, in contrast to the isolation of different clones expressing various levels of c-Kit as described in Chapter 4. The former method would therefore minimise any effects due to clonal variation.

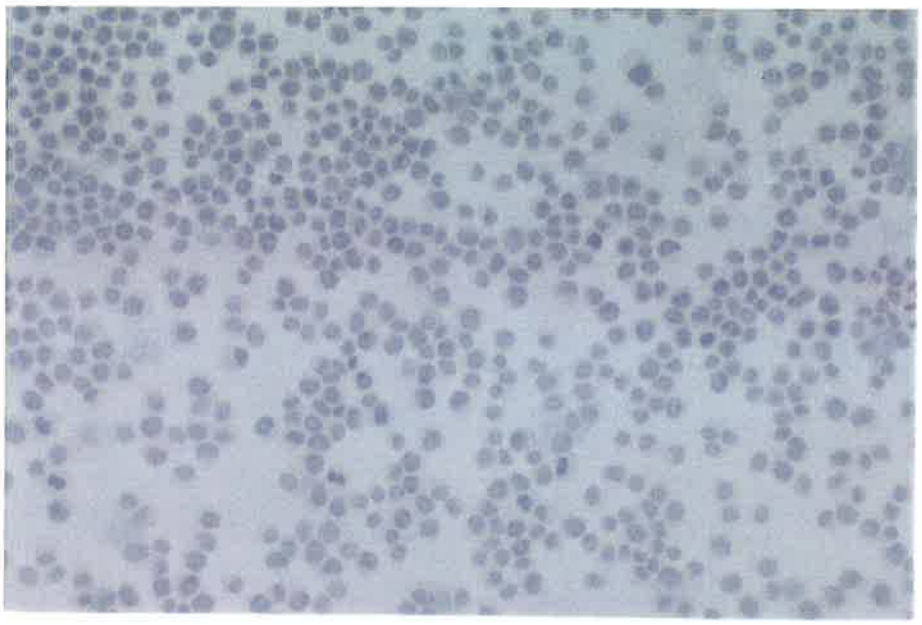
The pool of G418^R NIH/3T3 transfectants, NIH(pGC1.2), were analysed for c-Kit surface protein expression via indirect immunofluorescence (IIF) with either of the α-c-Kit

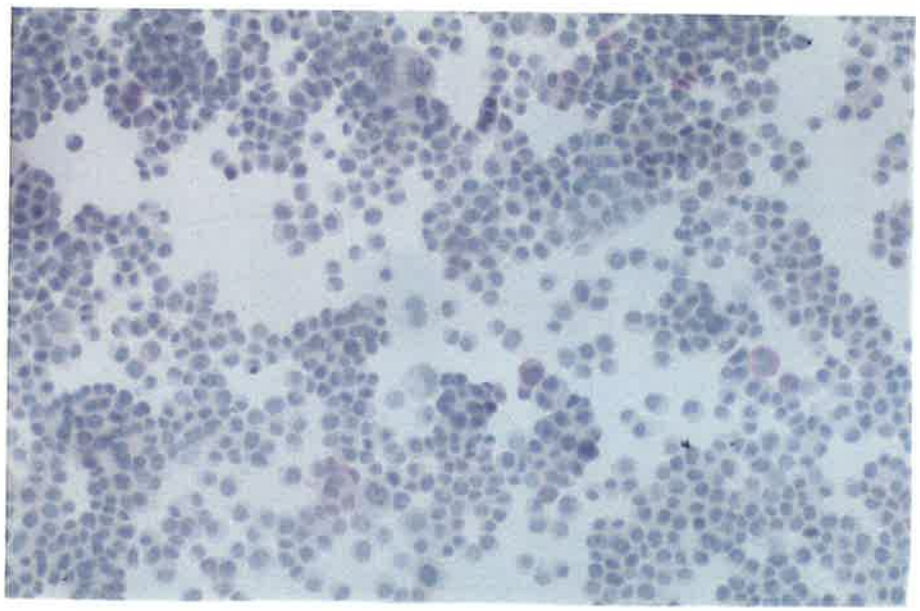
Figure 5.2.1: Composition of the pGC1.2 construct. The entire c-*kit* cDNA coding sequence (3 kb) (Yarden *et al.*, 1987a) was excised from the pBluescript(SK) (pBS(SK)) construct obtained from Immunex using Asp 718 - Not I. Klenow polymerase was used to fill in the ends and the fragment was cloned into the Bam HI site of the pRSV009/A+ vector (derivation described by Choo *et al.*, 1986). This placed c-*kit* under the control of the RSV LTR. A polyadenylation signal was derived from the sheep metallothionein (MT) gene. The vector also contained the *neo* resistance (neo^R) gene, to allow for selection of primary transfectants using cellular resistance to geneticin (G418); the murine dihydrofolate reductase (*DHFR*) gene for methotrexate resistance (Mtx^R), which was used to amplify the c-*kit* sequence under selective pressure; and the ampicillin resistance (amp^R) gene to allow for selection of E.*coli* containing the plasmid. Both the *DHFR* and neo^R genes are expressed from the promoter of the early region of the simian virus (SV40). The SV40 small T introns are also shown (__^__). The resulting pGC1.2 construct was 11.3 kb in size.


c-kit
BamHI
EcoRI
EcoRI
SalI
LTR
MT
AAAA
HindIII
PSV40L
PSV40E
ampR
DHFR
pRSV009/A+
BglII
PSV40L
PSV40E
neoR
HindIII
BglII

mAb, YB5.B8 or IDC3, binding being detected by a second stage α-IgG conjugated to fluorescein-isothiocyanate (FITC). Using this technique the pool of transfectants were found to be negative. It was possible that only a small fraction of the pool was expressing c-Kit making it difficult to detect these rare cells by this method. Cells were therefore stained by immunohistochemistry using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique (see section 2.2.5 for method), employing the same primary antibodies as above. This revealed that even though the cells were $G418^R$, on average only 5% of the cells were expressing detectable c-Kit protein (Figure 5.2.2). The rare c-Kit expressing cells were isolated from the negative pool population by immune rosetting (see section 2.2.6 for method). Dishes were seeded at low densities with the NIH(pGC1.2) pool of transfectants to allow for the formation of well-isolated colonies. Dishes were then incubated with a cocktail of 1DC3 and YB5.B8 mAb and binding of these mAb to the cells expressing c-Kit was detected using goat anti mouse coupled - red blood cells (GαM - RBC). Figure 5.2.3 shows a typical colony which has bound GαM - RBC. Twenty RBC-coated colonies were harvested from the pool population using cloning rings and placed under a second round of immune rosetting. Seven of the initial twenty colonies showed repetitive binding to the GαM - RBC. Colonies were again isolated via cloning rings, expanded, and their levels of c-Kit surface expression subsequently monitored via IIF. The c-Kit positive fractions of the cell populations were collected by IIF-activated cell sorting. Clones were isolated via limiting dilution from three of the seven populations which had bound GαM - RBC. 15 clones were isolated and were initially placed under methotrexate selection. NIH/3T3 untransfected cells express endogenous *DHFR* therefore an initial concentration of 100 nM Mtx was chosen as it excluded endogenous resistance. Transfected cells were cultured for two weeks in 100 nM Mtx and then a fraction of the cells were placed under 200 nM Mtx selection. The cells were made resistant to two fold increasing concentrations of Mtx every two weeks to a maximum of 1600 nM (refer also to section 2.8.2 for method). Higher concentrations greatly reduced the proliferation rate of the cells. Of the 15 clones placed under Mtx selection, NIH(C3A3) was selected for further analysis due to its ability to amplify c-Kit the most effectively (Figure 5.2.4). A comparison of the mean fluorescence intensities (mfi) of the unselected NIH(C3A3)

Figure 5.2.2: Immunohistochemical analysis of c-Kit protein expression by the NIH(pGC1.2) pool of infectants. Cells were cytocentrifuged onto glass slides, fixed and incubated with the α-c-Kit mAb 1DC3. Detection of 1DC3 binding to the cells was performed using a bridging rabbit α-mouse antibody, followed by an alkaline phosphatase-mouse α-alkaline phosphatase complex. Cells were then incubated in substrate, resulting in c-Kit expressing cells staining red. Cells were visualised under an Olympus microscope and photographed. Photograph at the top of the page represents NIH(pRSVOO9/A+) cells (10 x magnification); middle of page represents NIH(pGC1.2) cells (10 x magnification); and bottom of page represents NIH(pGC1.2) cells (20 x magnification).





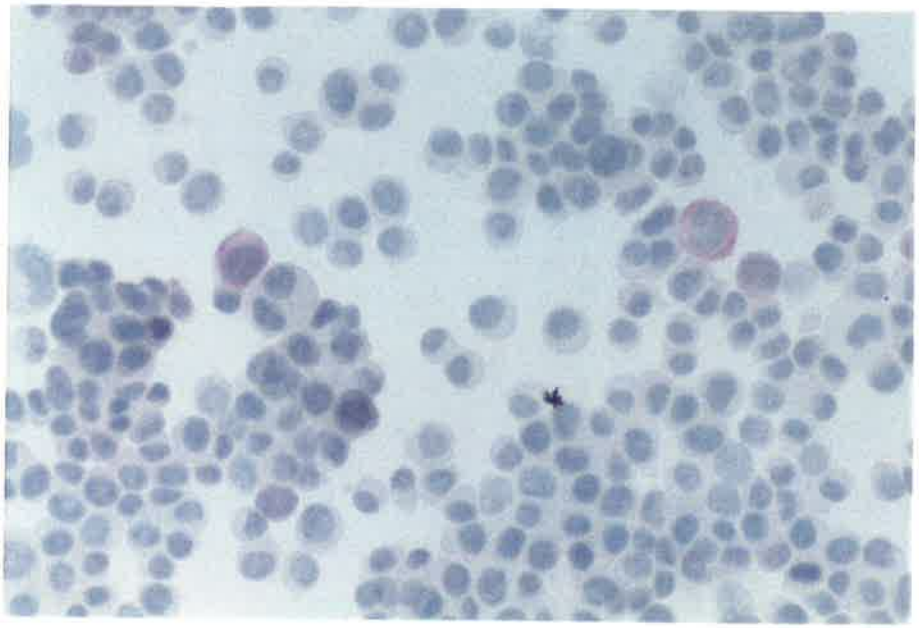
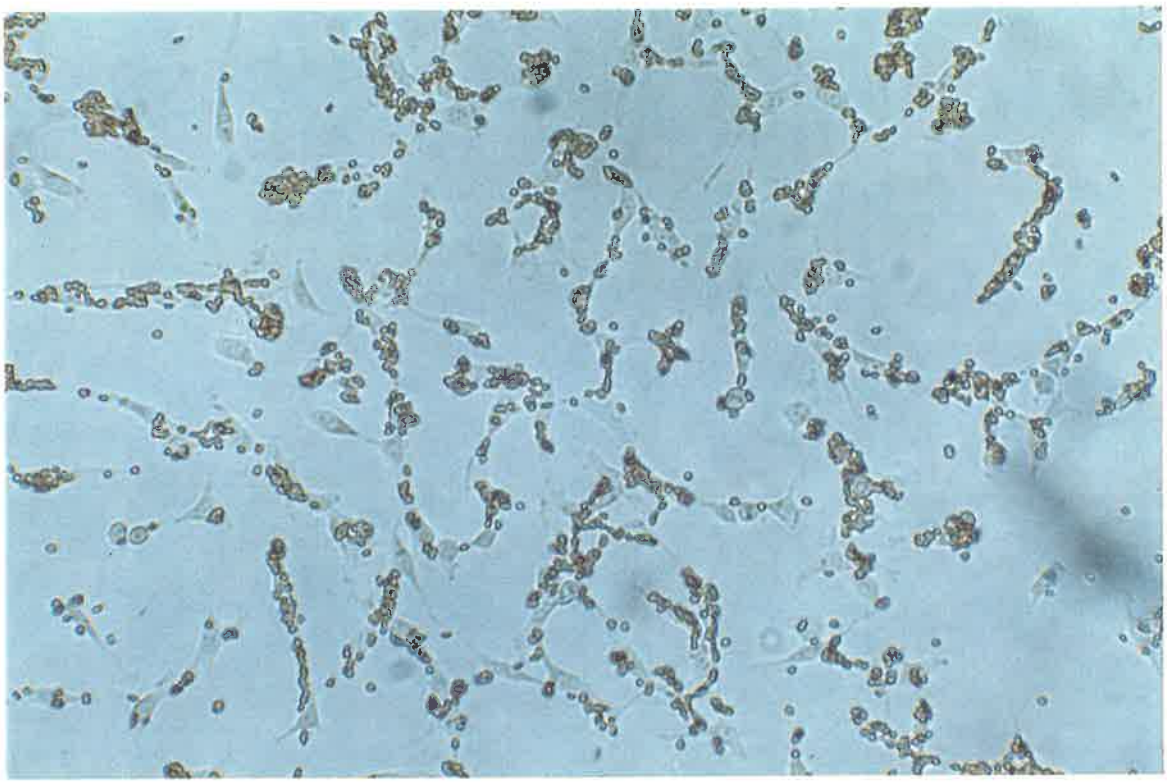


Figure 5.2.3: Detection of c-Kit expressing cells within the NIH(pGC1.2) pool via immune rosetting. Dishes containing isolated colonies were incubated with a cocktail of the α-c-Kit mAb, YB5.B8 and 1DC3. Binding of the antibodies to the cells was detected by subsequent binding of GαM coupled-RBC. Positive colonies were scored microscopically using an inverted Olympus microscope and representative colonies photographed. Photograph at top of page represents NIH(pRSVOO9/A^+) cells (4x magnification); bottom of page represents a typical RBC-coated NIH(pGC1.2) colony (10x magnification).



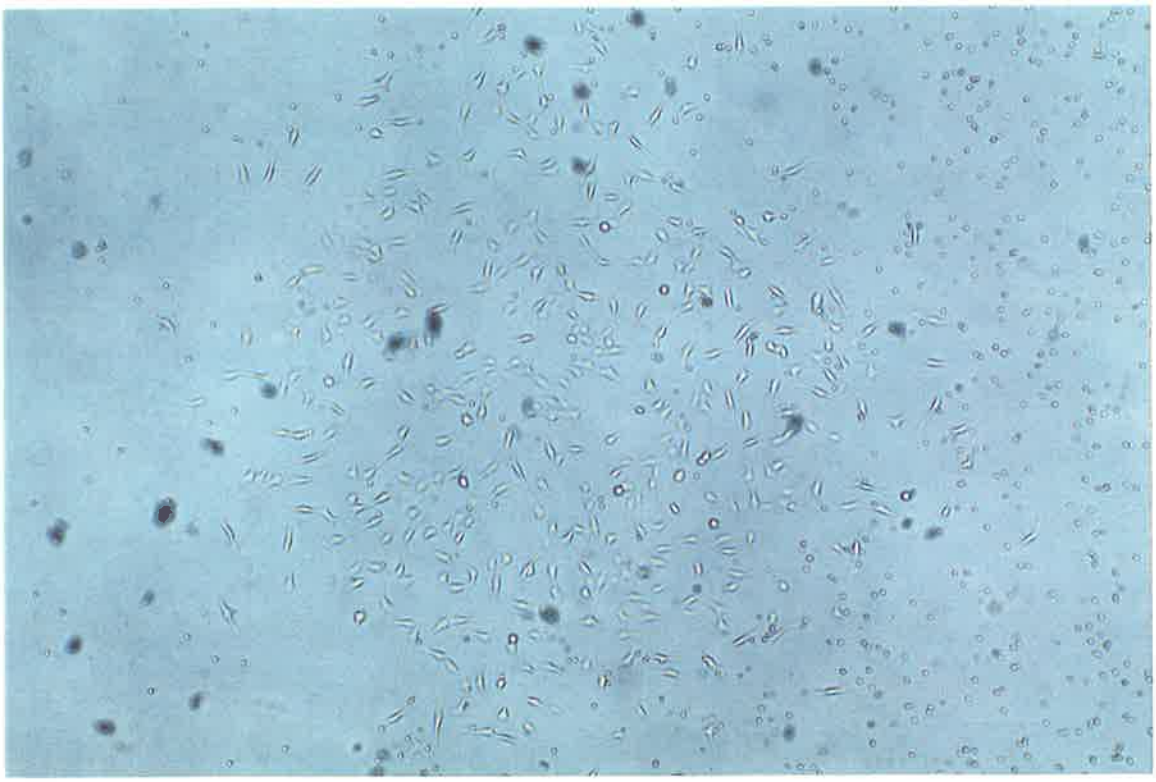
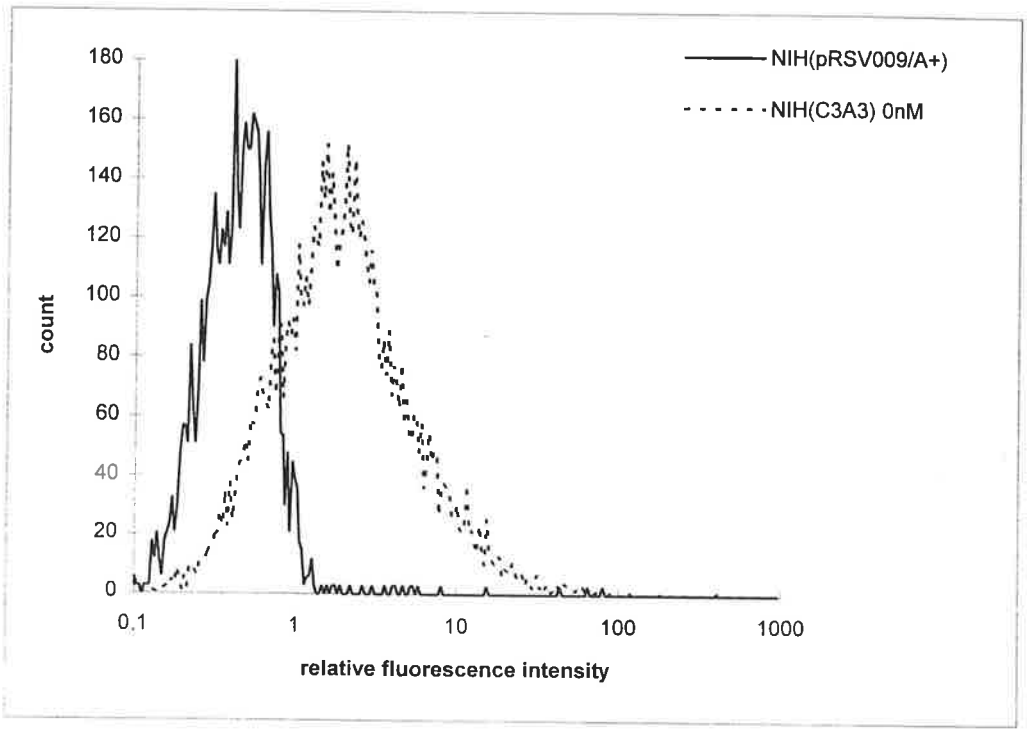
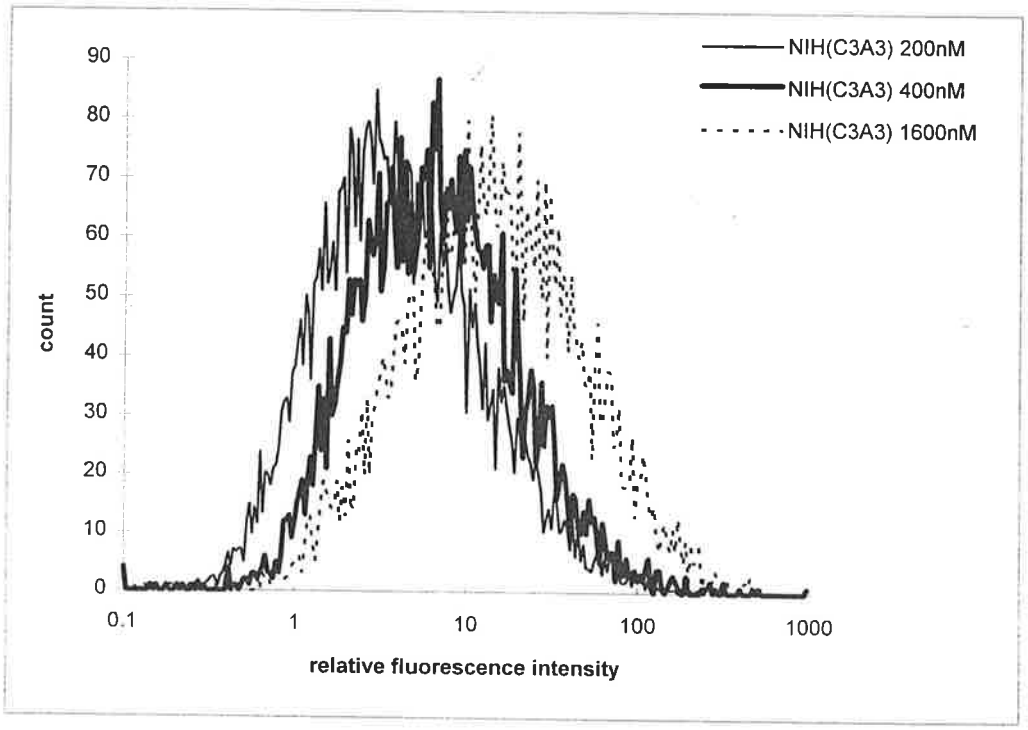


Figure 5.2.4: Expression of c-Kit surface protein by the NIH(C3A3) series of methotrexate resistant cells. Cells which had undergone selection in increasing concentrations of Mtx were labelled with the α-c-Kit mAb 1DC3. Binding was detected by IIF using a secondary fluorescein isothiocyanate (FITC) conjugated Ig and flow cytometric analysis.

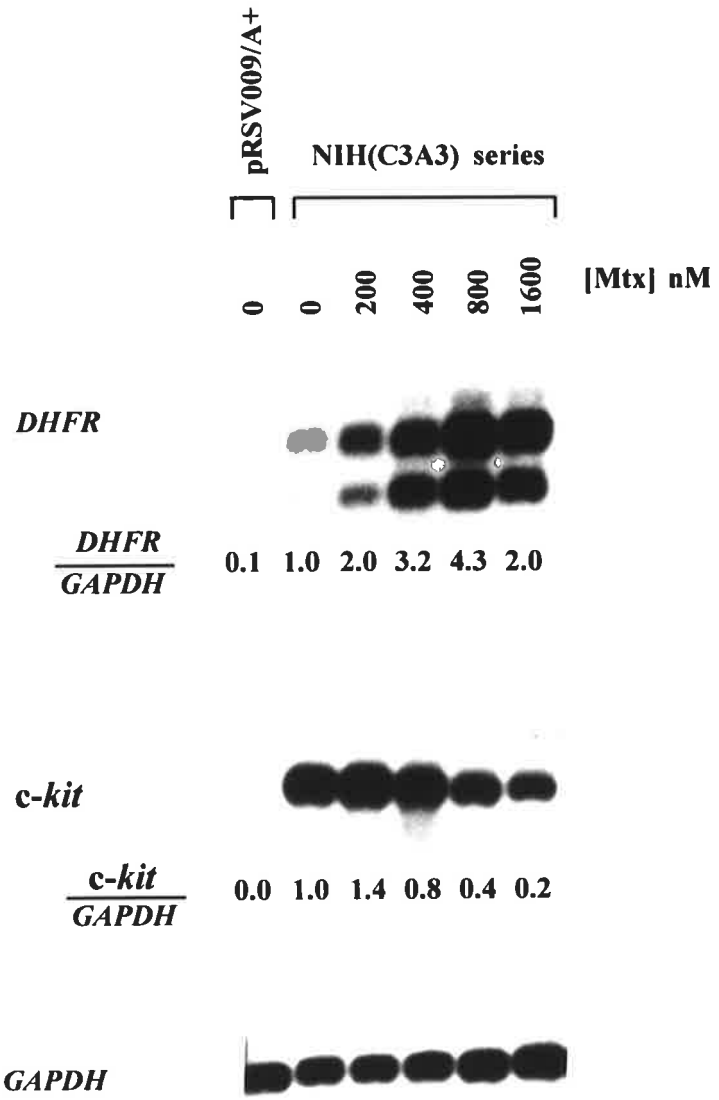

NIH(pRSV009/A+)
NIH(C3A3) 0nM
count
180
160
140
120
100
80
60
40
20
0
0.1
1
10
100
1000
relative fluorescence intensity


NIH(C3A3) 200nM
NIH(C3A3) 400nM
NIH(C3A3) 1600nM
count
90
80
70
60
50
40
30
20
10
0
0.1
1
10
100
1000
relative fluorescence intensity

cells with those resistant to 1600 nM Mtx demonstrated that a 8.7 fold increase in c-Kit protein expression was achieved. Control cells which had been transfected with the pRSV009/A$^+$ vector without the c-*kit* insert were also selected for resistance to 1600 nM Mtx.

Northern blot and PhosphorImager analysis demonstrated that the level of *DHFR* mRNA expression had increased in cells cultured in increasing concentrations of Mtx except those cells cultured in 1600 nM Mtx (Figure 5.2.5). The decrease in *DHFR* amplification in cells cultured in 1600 nM Mtx may indicate that maximal amplification had been achieved at around 800 nM Mtx. This is consistent with the observation that cells selected at higher levels displayed a reduced proliferation rate. Several experiments demonstrated that maximum c-Kit protein expression was obtained between 800 - 1600 nM Mtx and cells cultured in 6400 nM Mtx expressed lower levels than the latter. Kaufman *et al.*, (1985) has described similar results where protein amplification appeared to reach a threshold. Two major *DHFR* mRNA transcripts were detected (Figure 5.2.5) due to the association of the *DHFR* gene with the SV40 small T intron, resulting in the generation of 1.3 kb (spliced) and 1.9 kb (unspliced) sized transcripts (see Figure 5.2.1). Another larger transcript was also detected which may be an endogenous *DHFR* transcript. Lack of ectopic *DHFR* mRNA in the NIH(pRSV009/A+) cells was not surprising since this population of cells was a pool and not a clone and the cells had not undergone Mtx selection. Although NIH(pRSV009/A+) cells were made resistant to 1600 nM Mtx their slow proliferative rate made it difficult to obtain large quantities of cells to harvest mRNA from therefore mRNA was harvested from the original NIH(pRSV009/A+) pool. Lack of detectable endogenous or introduced *DHFR* in unselected cells by Northern blot analysis has been reported elsewhere (Choo *et al.*, 1986). Unexpectedly, the level of c-*kit* mRNA did not increase with increasing concentrations of Mtx but instead an inverse relationship appeared to exist between the levels of c-*kit* mRNA and c-Kit surface protein expression (although statistically this was not significant $R = -0.8$; $p > 0.05$; Spearman Rank Correlation test). A reproducible 1.6 fold decrease in c-*kit* mRNA expression was seen between cells resistant to 200 nM and 400 nM Mtx yet there was a 1.6 fold increase in *DHFR* expression between these two cell populations (Figure 5.2.5) and a 1.7 fold increase in c-Kit protein (comparing relative mfi). A c-*kit* transcript of 3.7 kb was detected by Northern blot

Figure 5.2.5: Expression of *DHFR* and c-*kit* mRNA levels in the NIH(C3A3) series of methotrexate amplified cells. Poly A^+ selected mRNA was harvested from the NIH(pRSV009/A^+) and NIH(C3A3) unselected cells, and NIH(C3A3) cells selected in 200-1600 nM Mtx. mRNA (2μg) was analysed by agarose gel electrophoresis and northern blot hybridisation. The nylon membrane was probed initially with the full length c-*kit(GNNK+S+)* cDNA (3 kb) and a 780 bp human *GAPDH* fragment obtained from pHcGAP. Signals were detected, visualised and digitised using a Molecular Dynamics PhosphorImager. Hybridisation signals were quantified and represented as c-*kit/GAPDH* ratios relative to the NIH(C3A3) unselected cells. The membrane was stripped and reprobed with a Hind III - Bgl II *DHFR* fragment excised from pRSV009/A^+, the signal quantified as above and represented as the *DHFR/GAPDH* ratio also relative to the NIH(C3A3) unselected cells.

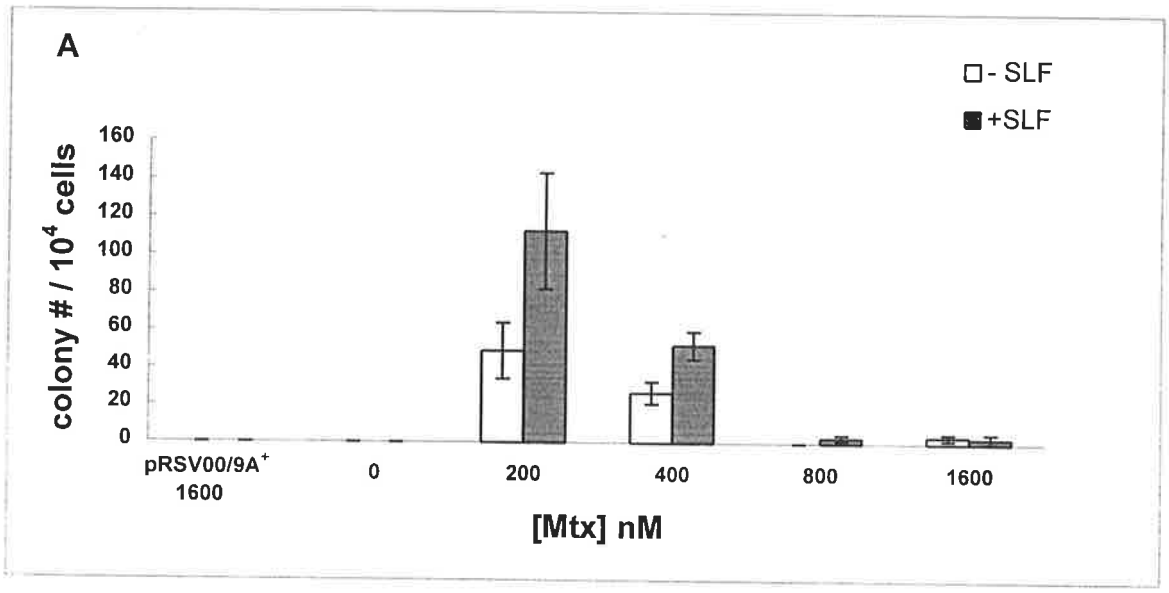

pRSV009/A+
NIH(C3A3) series
0
0
200
400
800
1600
[Mtx] nM
DHFR
DHFR / GAPDH
0.1 1.0 2.0 3.2 4.3 2.0
c-kit
c-kit / GAPDH
0.0 1.0 1.4 0.8 0.4 0.2
GAPDH

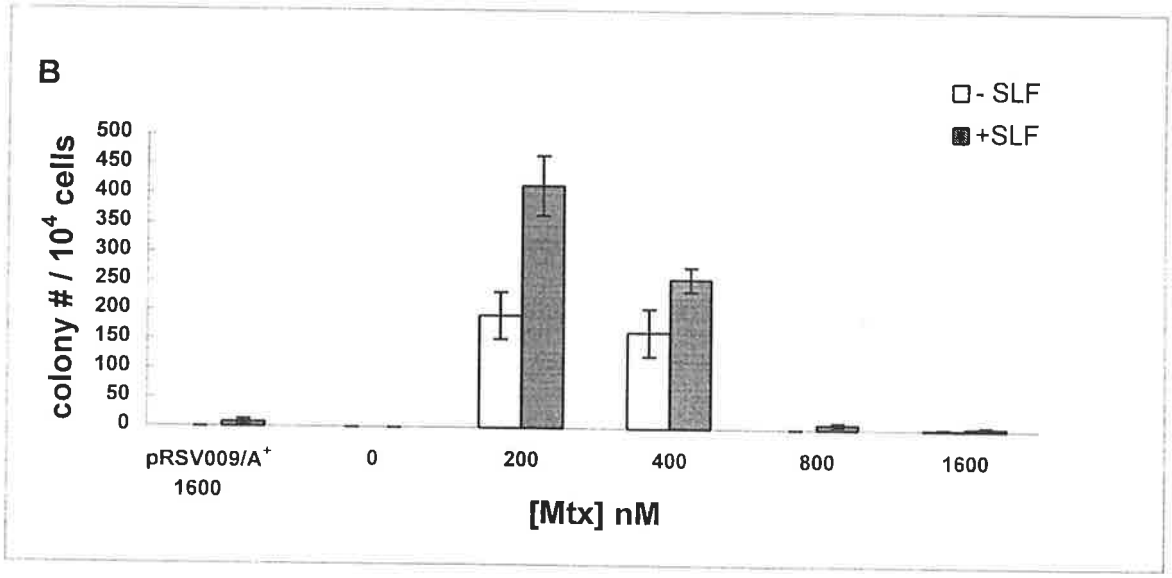
and hybridisation analysis (Figure 5.2.5) which corresponded to the size expected from this transcriptional unit.

NIH(C3A3) cells expressing increasing levels of surface c-Kit protein were plated in semi-solid agar to assess their ability to grow in an anchorage independent fashion. Figure 5.2.6 demonstrates that the unselected NIH(C3A3) cells and control cells NIH(pRSV009/A^{+})$_{1600\ nM}$ did not produce any colonies consisting of >50 cells in the presence or absence of 100 ng/ml human SLF (hSLF). Cells which had been selected to grow in 200 nM Mtx, NIH(C3A3)$_{200\ nM}$, were able to produce colonies in the absence and presence of factor at a plating efficiency of 0.5% and 1.13%, respectively, and clusters of 10-50 cells at 2% and 4.15%, respectively. These cells expressed 2.4 fold higher levels of surface c-Kit protein compared to the unselected NIH(C3A3) cells (comparison of relative mfi, Figure 5.2.4). Interestingly, NIH(C3A3)$_{400\ nM}$ cells produced colonies at a lower frequency than NIH(C3A3)$_{200\ nM}$ even though the level of c-Kit surface expression had increased by 1.7 fold (comparison of mfi) (Figure 5.2.6 and 5.2.4). Cells placed under greater than 400 nM Mtx selection which had shown a further increase in c-Kit protein expression (Figure 5.2.4) produced a negligible number of colonies. It therefore appeared that an inverse relationship also existed between c-Kit surface protein expression and anchorage independent growth in the selected NIH(C3A3) cells.

Many problems were associated with the DHFR amplification system such as: low frequency of c-Kit expressing cells within the G418^{R} pool, making isolation of these cells difficult; the long period of time required to obtain Mtx resistant cells expressing increasing levels of c-Kit; the possibility that the true effects of c-Kit overexpression were being masked by contributing effects of high Mtx concentrations; and the discrepancy in the levels of c-Kit protein compared to the levels of c-*kit* mRNA. Due to these problems it was decided to use a similar method to that described in Chapter 4 to obtain pool populations of cells expressing increasing levels of c-Kit surface protein. cDNAs encoding the various c-Kit isoforms were constructed and cloned into the retroviral vector pRUFMC1*neo* as described below.

Figure 5.2.6: Anchorage independent growth of the NIH(C3A3) series of methotrexate resistant cells. 10^4 cells were plated in 0.33% semi-solid agar in the presence or absence of 100 ng/ml hSLF. Colonies comprising of >50 cells (A) and 10-50 cells (B) were scored microscopically after 14 days. Data represented as the mean colony number ± S.E.M. of quadruplicate replicates.


A
- SLF
+SLF
colony # / 10^4 cells
160
140
120
100
80
60
40
20
0
pRSV00/9A+
1600
0
200
400
800
1600
[Mtx] nM


B
- SLF
+SLF
colony # / 10^4 cells
500
450
400
350
300
250
200
150
100
50
0
pRSV009/A+
1600
0
200
400
800
1600
[Mtx] nM

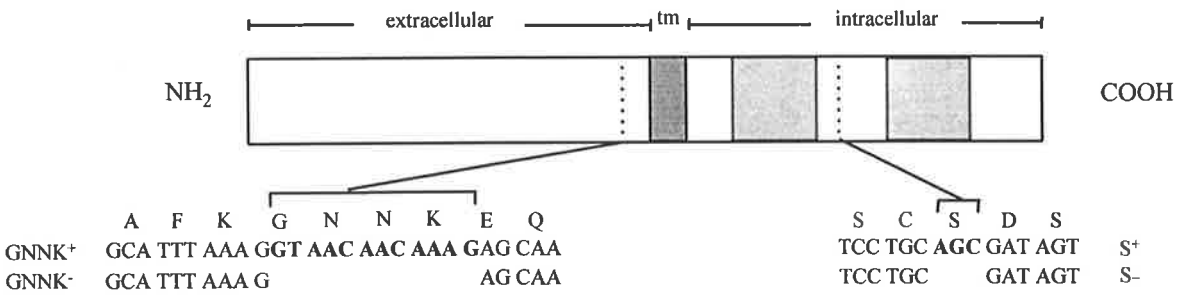
5.3 *Production of cDNAs encoding the GNNK+/- and S+/- c-Kit isoforms*

Three other c-*kit* cDNA constructs which possessed a 12 bp deletion/insertion of nucleotides 1550-1561 encoding the 4 amino acids GNNK and/or a 3 bp deletion/insertion of nucleotides 2164-2166 encoding a serine were generated (Figure 5.3.1 A). The cDNA constructs c-*kit(GNNK+S-)*, c-*kit(GNNK-S+)* and c-*kit(GNNK-S-)* were generated by polymerase chain reaction (PCR) site directed mutagenesis using the available 3 kb c-*kit(GNNK+S+)* cDNA insert in the pBS(SK) vector as a template. To generate the c-*kit(GNNK-S+)* cDNA, primers 796 (anti-sense) and 797 (sense) (refer to Table 2.5.2.1 for sequence and locations) which flanked the area to be deleted were used to amplify a 6 kb PCR product using the conditions described in Section 2.8.1(b). The 6 kb PCR product comprised of the c-*kit* sequence, minus the 12 bp encoding GNNK, and the pBS(SK) vector (refer to Figure 5.3.1 B). The entire c-*kit* product was sequenced (refer to section 2.6 for method) using a selection of primers listed in Table 2.5.2.1 and corresponded to the published sequence (Yarden *et al.*, 1987a), except for the removal of the 12 bp and also a base change at position 2415 where a C was replaced by a T, however this change did not alter the amino acid encoded (Ile). Sequencing of the template c-*kit(GNNK+S+)* cDNA across this region revealed that this base change was also found in the template and therefore no errors had been introduced during PCR amplification of the c-*kit(GNNK-S+)* product.

Similarly, deletion of the codon for serine at position 2164-2166 was achieved using the primers 1002 (anti-sense) and 1003 (sense) (refer to Table 2.5.2.1 for sequence of primers and Figure 5.3.1 B). A Dra II-Bgl II fragment (~220 bp) containing the serine deletion was excised from the 6 kb PCR-generated product and substituted into the c-*kit(GNNK+S+)* and c-*kit(GNNK-S+)* cDNAs to generate c-*kit(GNNK+S-)* and c-*kit(GNNK-S-)*, respectively. All the resulting isoforms were sequenced around the deletions/insertions and also around the areas of manipulation. All sequences verified that the four cDNAs encoding the c-Kit isoforms had been generated correctly (Figure 5.3.2).

The c-*kit* cDNAs encoding the various isoforms were excised from pBS(SK) with Asp 718 - Not I, end-filled and cloned into the Hpa I site of the retroviral vector pRUFMC1*neo* (refer to methods described in section 2.4) to generate pRUF(*GNNK+S+*), pRUF(*GNNK-S+*),

Figure 5.3.1: Diagramatic representation of the production of the *c-kit(GNNK+/-S+/-)* variants. (A) The insertion/deletion of the 4 amino acids Gly-Asn-Asn-Lys is located at position 510-513 (in accordance with the published sequence by Yarden *et al.*, 1987a). The insertion/deletion of the serine residue is located at position 715 within the interkinase domain. Hatched area indicates the transmembrane domain (tm) and the dotted areas indicate the split kinase domain of the c-Kit protein. Lower diagram represents the exon/intron and intron/exon junction sequences (underlined) respectively involved in generating the alternate splice variants c-*kit(GNNK+/-)* and c-*kit(S+/-)*. Diagrams are a compilation of those published by Reith *et al.*, (1991), Crosier *et al.*, (1993) and Vandenbark *et al.*, (1992).



510 511 512 513

exon 9+ .....GCA TTT AAA GGT AAC AAC AAA Ggt ata

exon 9- .....GCA TTT AAA Ggt aac aac aaa ggt ata

715 716

tca tct tct ccc agC AGC GAT..... exon 15+

tca tct tct ccc agc agC GAT..... exon 15-

Figure 5.3.1: Diagramatic representation of the production of the *c-kit(GNNK+/-S+/-)* variants. (B) c-*kit(GNNK+S+)* cDNA cloned between the Asp 718 - Not 1 sites of the pBluescript(SK) (pBS(SK)) was used as template for the amplification of *c-kit(GNNK-S+)*. Primers 796 (anti-sense) and 797 (sense) (refer to Table 2.5.2.1 for sequences) flanking the 12 bp to be deleted were used to amplify a 6 kb product (pBS(SK)+c-*kit*) by the PCR. The 5' ends were phosphorylated and the ends ligated to generate *c-kit(GNNK-S+)* harboured in pBS(SK). Similarly the 3 bp encoding the serine amino acid were removed from the template using the flanking primers 1002 (anti-sense) and 1003 (sense) (refer to Table 2.5.2.1 for sequences). A Dra II - Bgl II fragment containing the deleted 3 bp was excised from the PCR product and substituted into the c-*kit(GNNK+/-)* containing cDNAs. The above manipulations resulted in the construction of 4 c-*kit* cDNAs **1.** c-*kit(GNNK+S+)*, **2.** c-*kit(GNNK-S+)*, **3.** c-*kit(GNNK-S-)* and **4.** c-*kit(GNNK+S-)*.

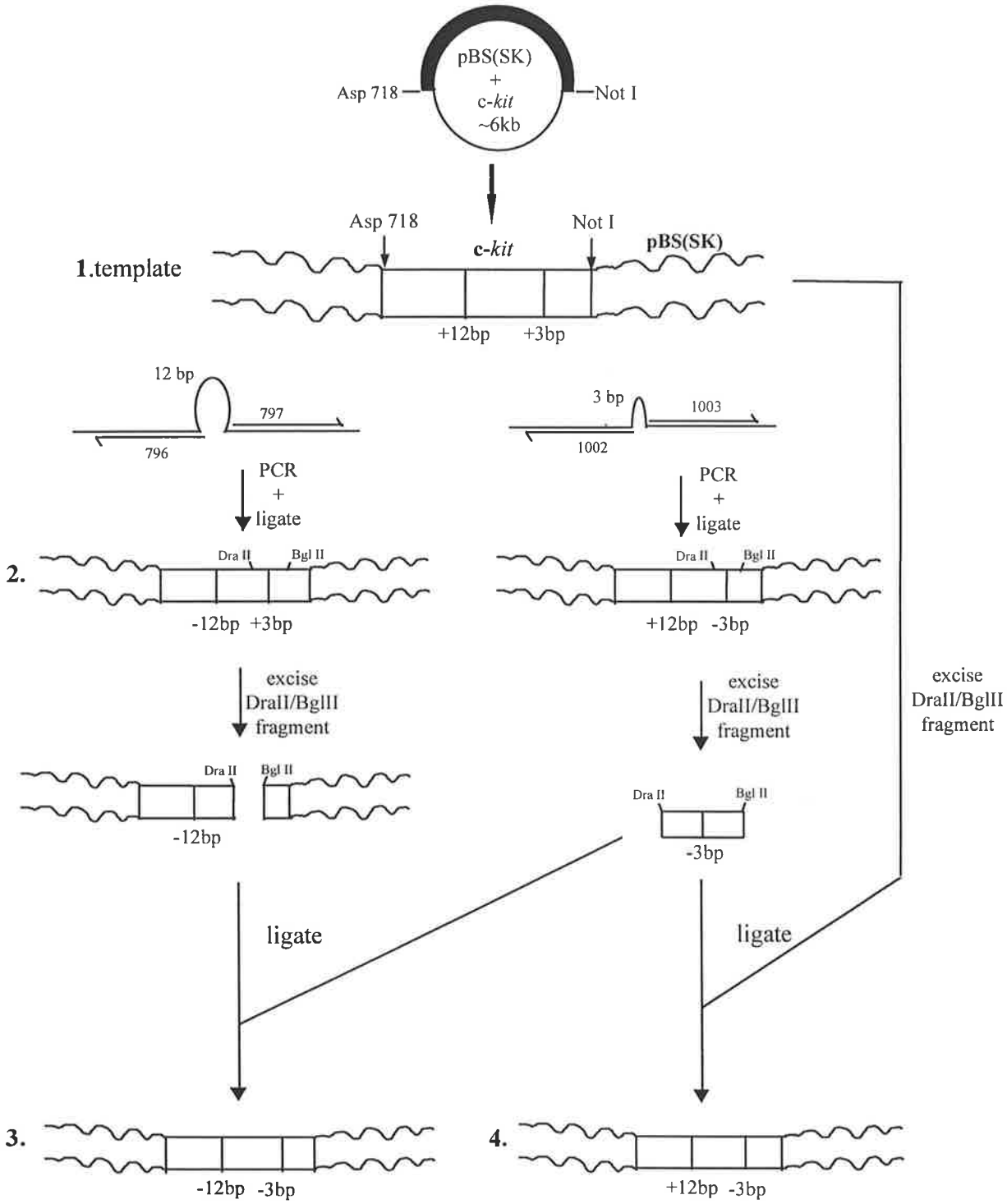
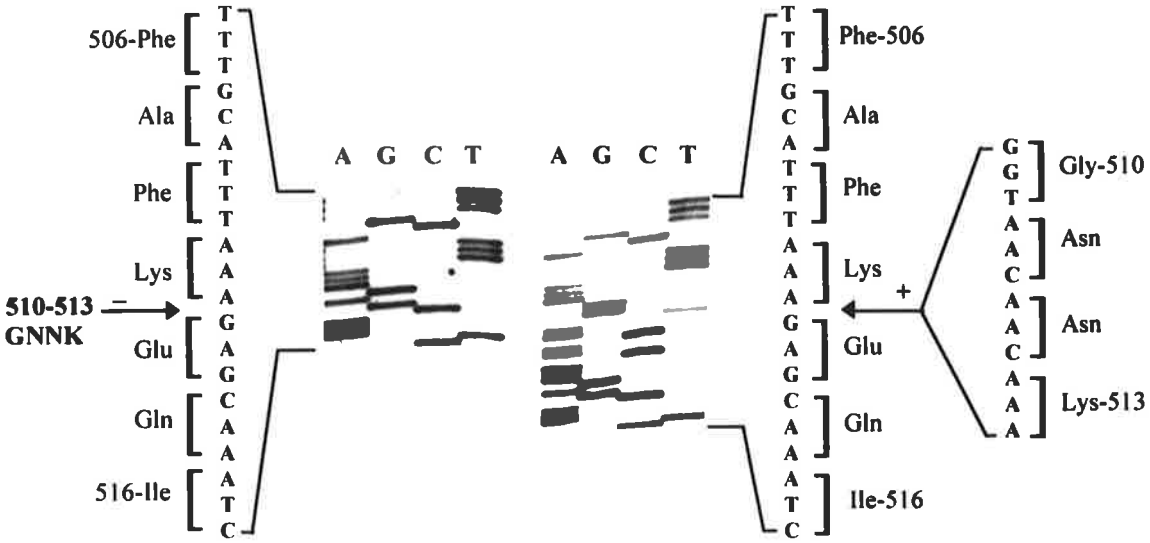

pBS(SK)
+
c-kit
~6kb
Asp 718
Not I
1.template
Asp 718
Not I
c-kit
pBS(SK)
+12bp
+3bp
12 bp
797
796
3 bp
1003
1002
PCR
+
ligate
PCR
+
ligate
2.
Dra II
Bgl II
-12bp +3bp
Dra II
Bgl II
+12bp -3bp
excise
DraII/BglII
fragment
excise
DraII/BglII
fragment
excise
DraII/BglII
fragment
Dra II
Bgl II
-12bp
Dra II
Bgl II
-3bp
ligate
ligate
3.
-12bp -3bp
4.
+12bp -3bp

Figure 5.3.2: Sequence analysis of the c-*kit(GNNK+/-S+/-)* cDNAs. The cDNAs encoding the c-Kit isoforms were sequenced around the areas containing **(A)** the 12 bp deletion/insertion encoding GNNK (510-513) and **(B)** the 3 bp insertion/deletion encoding the Ser residue (715), using the primers 1142 and 1004, respectively (refer to Table 2.5.2.1 for sequences and binding locations of primers).

A


506-Phe
Ala
Phe
Lys
510-513
GNNK
Glu
Gln
516-Ile
A G C T
A G C T
Phe-506
Ala
Phe
Lys
Glu
Gln
Ile-516
+
Gly-510
Asn
Asn
Lys-513

B

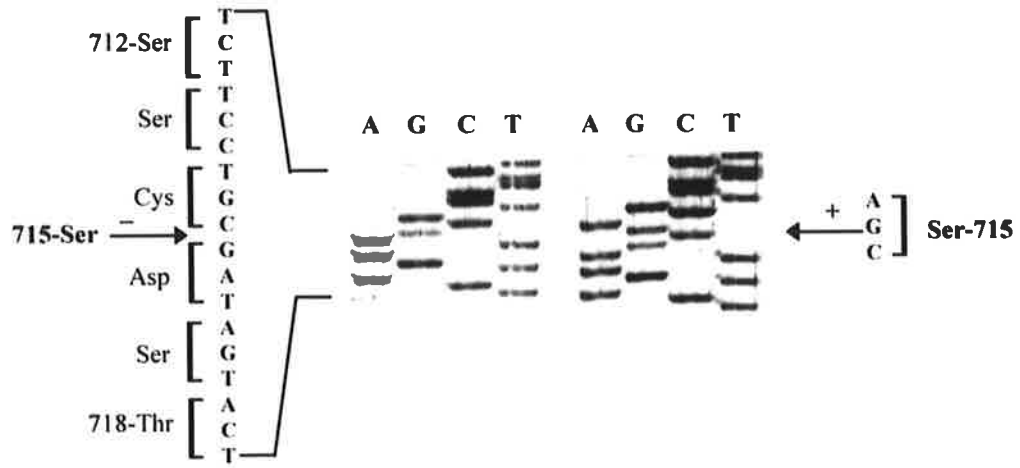
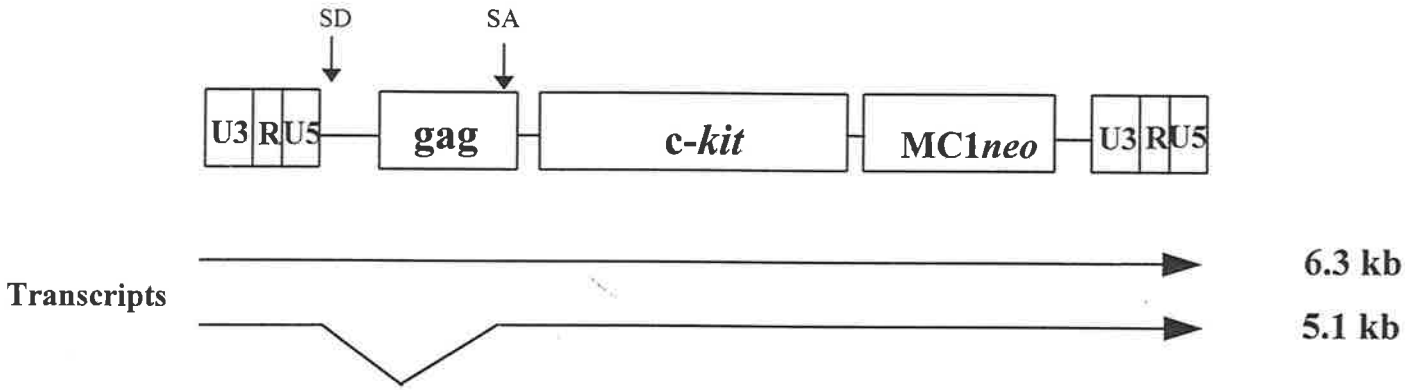

712-Ser
Ser
Cys
715-Ser
Asp
Ser
718-Thr
A G C T
A G C T
+
Ser-715

Figure 5.3.3: Composition of the pRUF(GNNK+/-S+/-) retroviral vectors. cDNAs encoding the c-Kit isoforms were inserted into the Hpa I site of the pRUFMC1*neo* vector. Derivation of the pRUFMC1*neo* vector which contains 5' and 3' LTRs derived from the myeloproliferative sarcoma virus has been previously described by Rayner & Gonda, (1994). Splice donor (SD) and splice acceptor (SA) sites are indicated by arrows. The spliced and unspliced transcripts, including the c-*kit* sequence, and their expected sizes are shown.

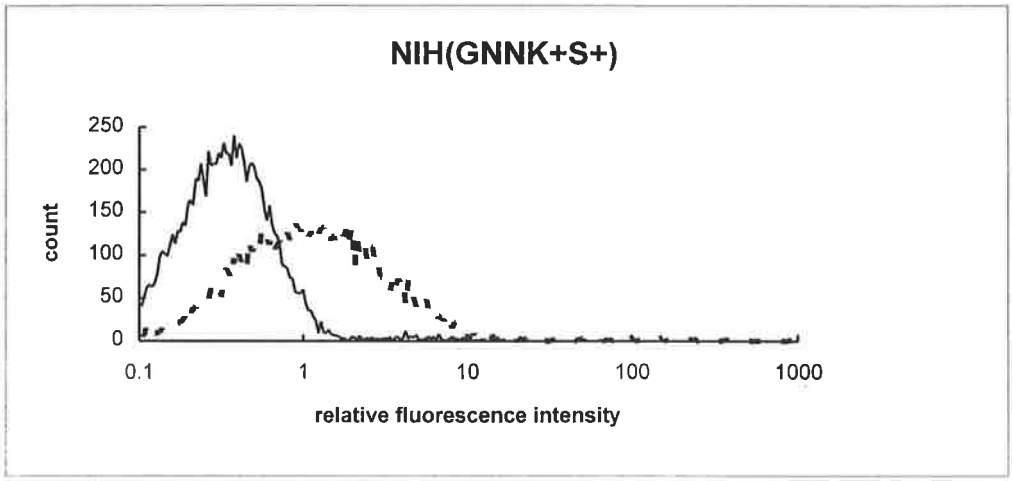

SD
SA
U3
R
U5
gag
c-kit
MC1neo
U3
R
U5
Transcripts
6.3 kb
5.1 kb

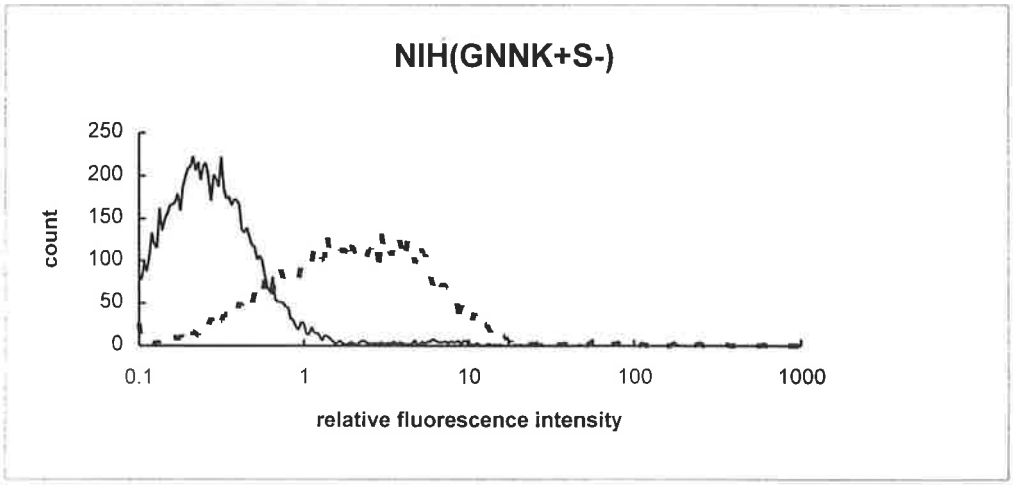
pRUF(*GNNK+S-*), and pRUF(*GNNK-S-*) (see Figure 5.3.3). pRUFMC1*neo* was used instead of pZenneo (as described in Chapter 4) as preliminary data indicated that the former vector was more efficient at infecting haemopoietic cells (Drs. T. Gonda and J. Rayner, unpublished), which we also intended doing (described in Chapter 6).

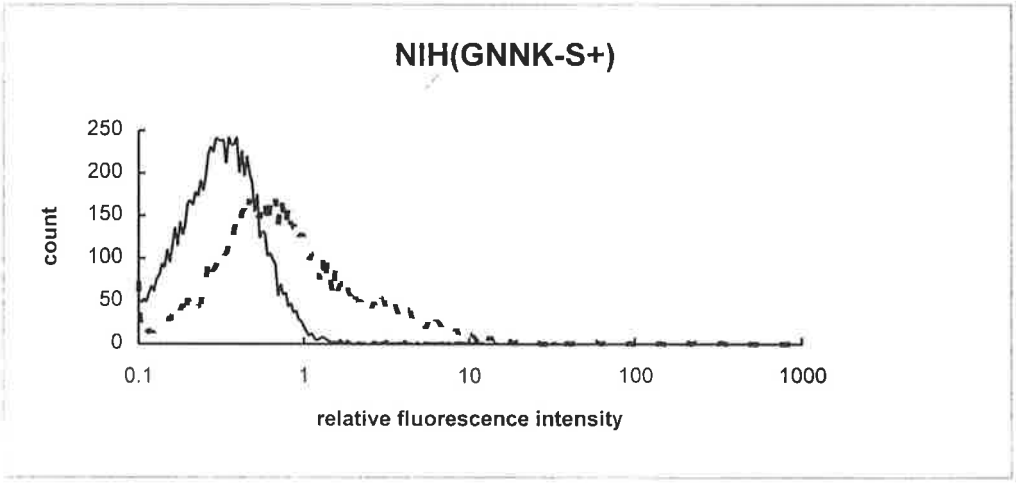
5.4 *Expression of the c-kit isoforms in the early passage NIH/3T3 cells*

The virus packaging cell line, ψ2, was transfected via the calcium phosphate method (see section 2.8.3 for method) with each of the pRUF*kit* constructs and also with the RUFMC1*neo* vector minus the c-*kit* insert as a control. The virus packaging cell lines generated were designated ψ2(*GNNK+S+*), ψ2(*GNNK+S+*), ψ2(*GNNK+S+*), ψ2(*GNNK+S+*) and ψ2(RUFMC1*neo*). Diluted virus containing supernatant from $G418^R$ pools of the ψ2 cells were used to infect the early passage (ep) NIH/3T3 cell line (refer to section 2.8.4 for method). The viral titres of these supernatants ranged from 10^5 - 6 x 10^5 cfu/ml. $G418^R$ epNIH/3T3 infected cells were screened for surface c-Kit expression by IIF using the α-c-Kit mAb 1DC3 and binding detected with a phycoerythrin (PE)-labelled second-stage α-IgG. Figure 5.4.1 demonstrates that all the c-Kit isoforms were expressed by the epNIH/3T3 cells except for c-Kit(GNNK-S-). There was variation in the level of c-Kit expression obtained between the isoforms, the GNNK+ containing isoforms appeared to be more abundantly expressed than GNNK-; this difference did not correlate with differences in viral titre. In order to compare the transforming potential of these isoforms, pools of cells expressing comparable levels of c-Kit were obtained by IIF-activated cell sorting. Cells were collected from three sort regions (the top 2% and 5% and the 10% preceding the top 5%, with respect to c-Kit expression) for each of the c-Kit isoform-expressing epNIH/3T3 cell pools. The resulting populations were designated epNIH(GNNK+/-S+/-).10, epNIH(GNNK+/-S+/-).5 and epNIH(GNNK+/-S+/-).2 and expressed c-Kit at various levels which allowed for comparisons between epNIH/3T3 cells expressing comparable levels of the different isoforms (Figure 5.4.2 and refer to Table 5.1). Unfortunately expression of the c-Kit(GNNK-S-) isoform was not detectable by IIF (Figure 5.4.1) nor by APAAP staining, using a panel of 8 α-c-Kit mAb which bind to distinct epitopes of the NH_2-terminus of the

Figure 5.4.1: c-Kit surface protein expression on primary epNIH(GNNK+/-S+/-) infectants. Cells were labelled with the α-c-Kit mAb, 1DC3 (·····) or an isotype-matched negative control mAb, 3D3.3 (____). Binding was detected using a second stage PE-conjugated Ig reagent and flow cytometric analysis.


NIH(GNNK+S+)
count
250
200
150
100
50
0
0.1
1
10
100
1000
relative fluorescence intensity


NIH(GNNK+S-)
count
250
200
150
100
50
0
0.1
1
10
100
1000
relative fluorescence intensity


NIH(GNNK-S+)
count
250
200
150
100
50
0
0.1
1
10
100
1000
relative fluorescence intensity

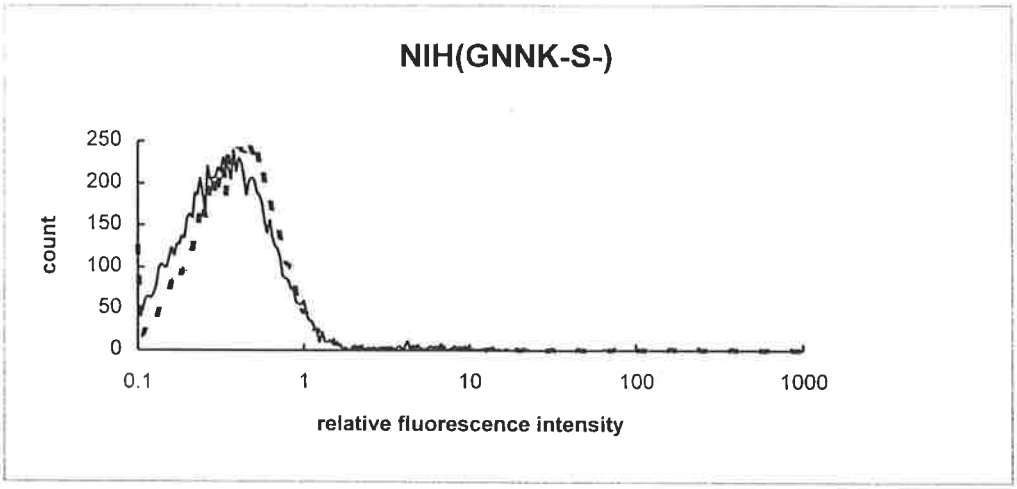
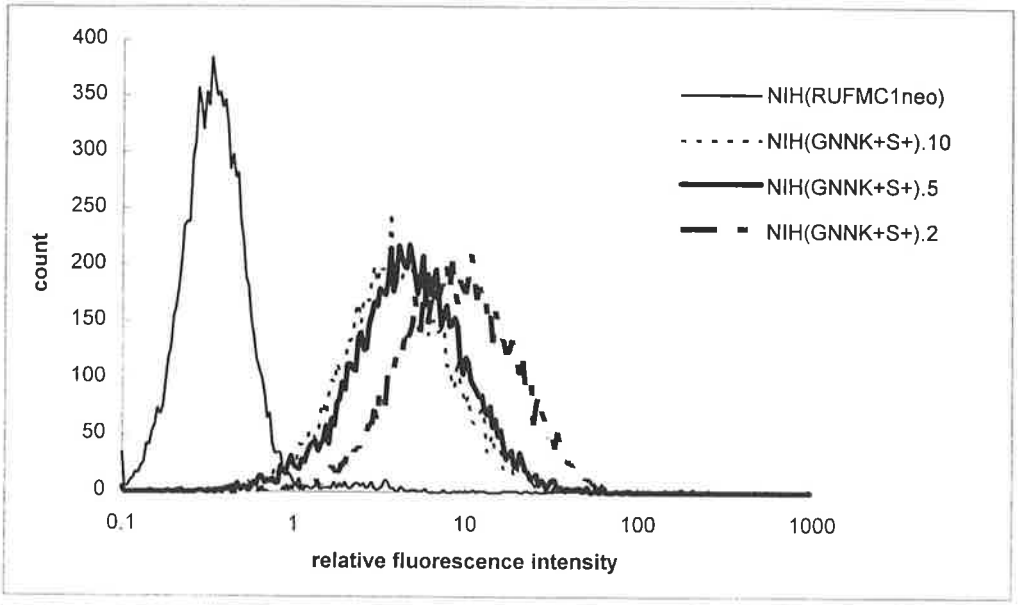
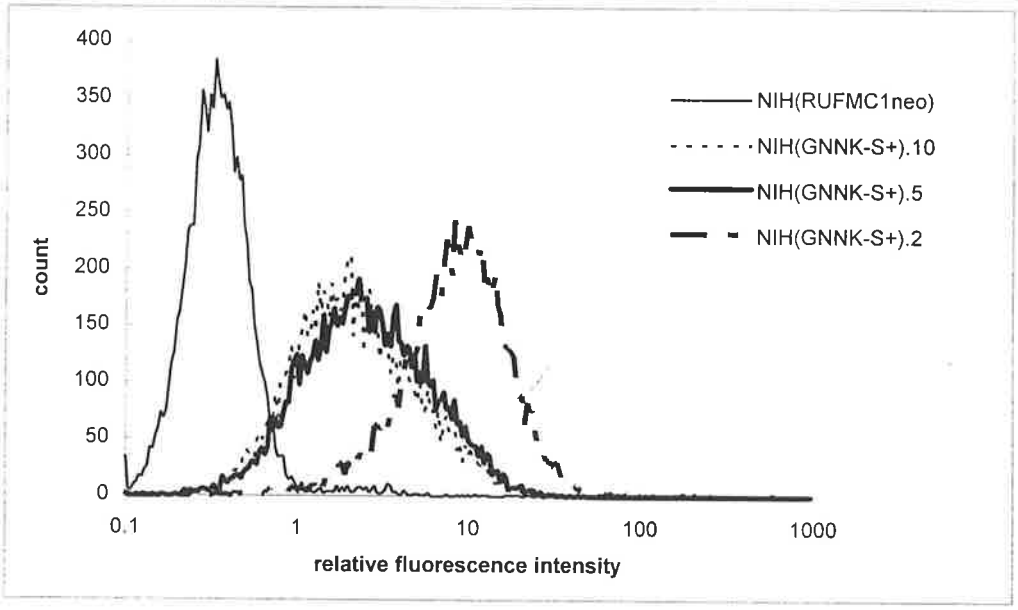
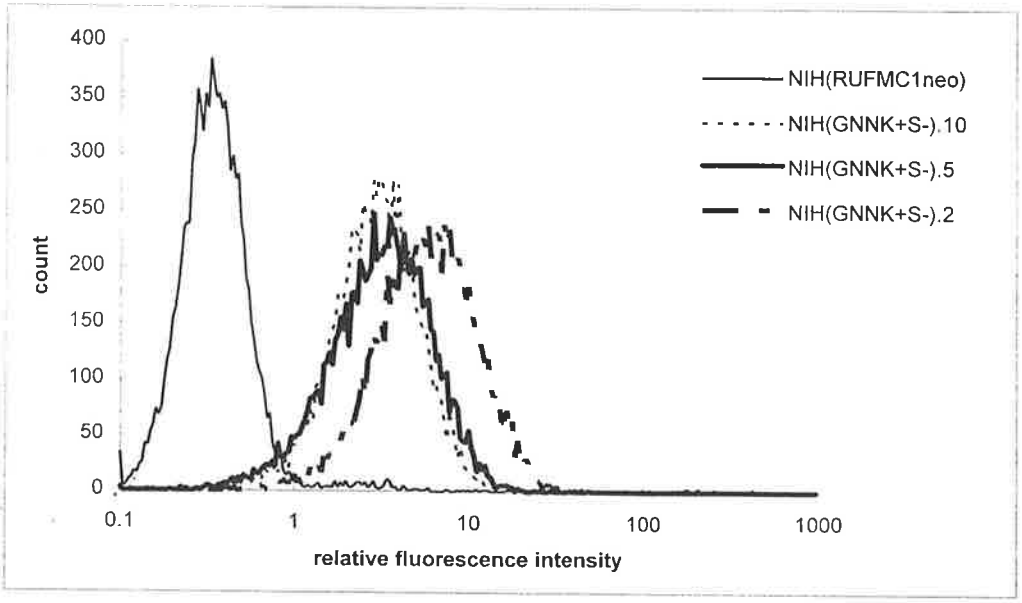

NIH(GNNK-S-)
count
250
200
150
100
50
0
0.1
1
10
100
1000
relative fluorescence intensity

Figure 5.4.2: c-Kit surface protein expression on selected epNIH(GNNK+/-S+/-) cells. Cell populations expressing increasing levels of surface c-Kit protein were selected from the primary epNIH/3T3 infectants by fluorescence-activated cell sorting, as described in the text, and expanded in culture. The histograms represent the expression of c-Kit(GNNK+/-S+) and c-Kit(GNNK+/S-) on the expanded populations detected by the α-c-Kit mAb, 1DC3, and a second stage PE-conjugated Ig reagent and analysed by flow cytometry. Control epNIH(RUFMC1*neo*) cells were also stained with 1DC3.


400
350
300
250
200
150
100
50
0
count
0.1
1
10
100
1000
relative fluorescence intensity
NIH(RUFMC1neo)
NIH(GNNK+S+).10
NIH(GNNK+S+).5
NIH(GNNK+S+).2

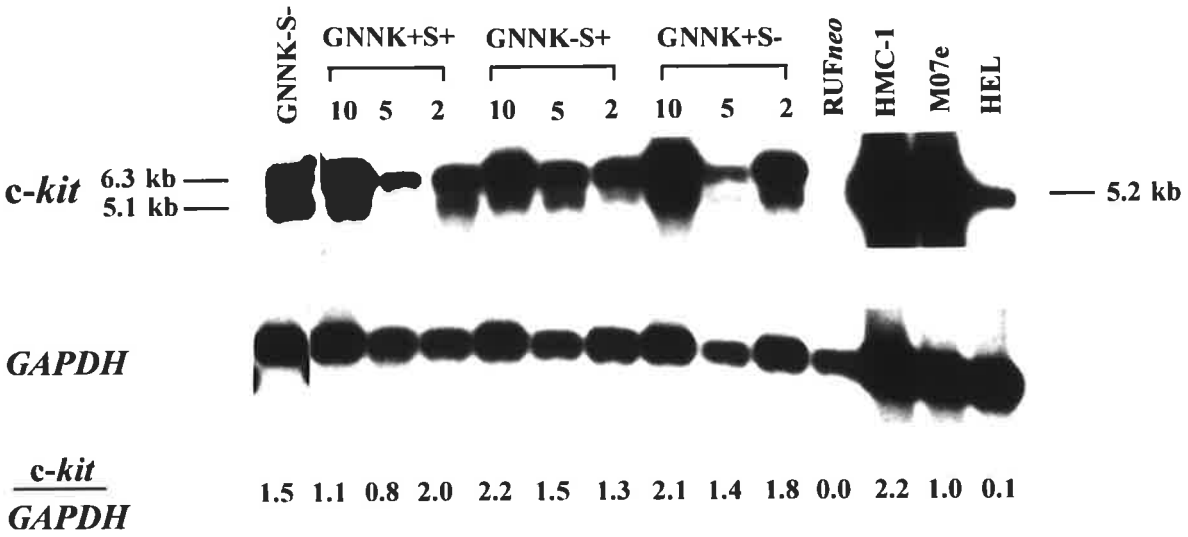

400
350
300
250
200
150
100
50
0
count
0.1
1
10
100
1000
relative fluorescence intensity
NIH(RUFMC1neo)
NIH(GNNK-S+).10
NIH(GNNK-S+).5
NIH(GNNK-S+).2


400
350
300
250
200
150
100
50
0
count
0.1
1
10
100
1000
relative fluorescence intensity
NIH(RUFMC1neo)
NIH(GNNK+S-).10
NIH(GNNK+S-).5
NIH(GNNK+S-).2

receptor. Further attempts were made to obtain expression of this isoform by reinfection and also by using a different c-*kit*(*GNNK-S-)* cDNA clone, however all the populations generated were $G418^R$ but did not express c-Kit protein although they did express c-*kit* mRNA (see below).

The number of receptors expressed on the surface of the epNIH/3T3 c-Kit infectants were also determined by quantitative IIF. Commercially available QIFIKIT® beads conjugated with known quantities of a murine monoclonal IgG_{2A} antibody were used to construct a standard curve as described in section 2.2.4. The lower level of expression obtained was about 3×10^3 and the highest ~ 1.9×10^4 receptors/cell (refer to table 5.1). The human cell line, HEL, (~2.6×10^4 receptors/cell) was used as a standard between repeated experiments. The level of c-*kit* mRNA expressed by these cells was also determined by Northern blot and PhosphorImager analysis using *GAPDH* mRNA levels as a control for comparative loading. Two transcripts of 6.3 and 5.1 kb in size were detected which correlated with the unspliced and spliced transcripts, respectively (represented in Figure 5.3.3). The human megakaryocytic cell line, M07e, was used as a reference for comparing c-*kit* mRNA levels between the epNIH/3T3 cells expressing the different c-*kit* isoforms and several leukaemic cell lines. Figure 5.4.3 demonstrates that the levels of c-*kit* mRNA did not correlate with the levels of surface c-Kit protein expressed by the epNIH/3T3 infectants (the latter were determined by IIF at the time of mRNA harvest and were equivalent to the levels represented in Table 5.1). However as for the NIH(C3A3) series of cells described in section 5.2 an inverse relationship between c-*kit* mRNA and c-Kit protein expression appeared to exist (although not statistically significant $R = -0.55$; $p > 0.05$; Spearman Rank Correlation test). Similar results were obtained when β- *actin* was used as an internal control for comparative loading (data not shown). However the leukaemic cell lines, HEL, M07e and HMC-1 progressively demonstrated an increase in c-*kit* mRNA levels as expected from their c-Kit surface protein expression (Cole *et al.*, 1996). Interestingly, the epNIH(GNNK-S-) cells expressed c-*kit* mRNA at levels comparable to epNIH(GNNK-S+).5 (Figure 5.4.3), yet failed to express protein as described above. Due to the paradox between c-*kit* mRNA and protein levels, subsequent references to c-Kit expression use the level of surface c-Kit protein.

Figure 5.4.3: Expression of c-*kit* mRNA levels in the epNIH(GNNK+/-S+/-) infectants. Poly A^+ selected mRNA (2μg) was analysed by agarose gel electrophoresis and northern blot hybridisation. The nylon membrane was probed with the full length c-*kit(GNNK+S+)* cDNA (3 kb) and a 780 bp human *GAPDH* fragment obtained from pHcGAP. Signals were detected, visualised and digitised using a Molecular Dynamics PhosphorImager. Hybridisation signals were quantified and represented as c-*kit/GAPDH* ratios relative to the M07e signal. The 6.3 and 5.1 kb bands indicated correspond to the transcripts generated fom the pRUFMC1*neo* constructs containing the c-*kit* genes while the 5.2 kb band corresponds to the normal c-*kit* mRNA produced in human cells.


GNNK-S-
GNNK+S+
GNNK-S+
GNNK+S-
10 5 2 10 5 2 10 5 2
RUFneo
HMC-1
M07e
HEL
c-kit
6.3 kb
5.1 kb
5.2 kb
GAPDH
c-kit / GAPDH
1.5 1.1 0.8 2.0 2.2 1.5 1.3 2.1 1.4 1.8 0.0 2.2 1.0 0.1

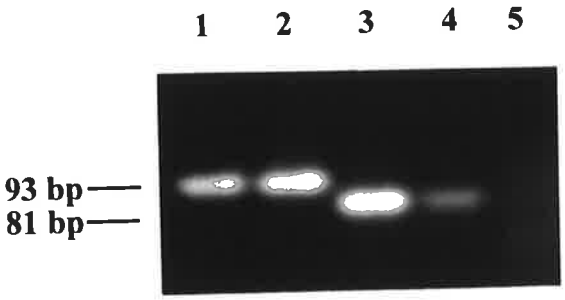
Verification of the c-Kit isoform expressed by the epNIH/3T3 infectants was performed prior to the commencement of functional studies. cDNA was generated from the cells expressing the different isoforms by reverse transcription and diagnostic restriction endonuclease digestion of PCR amplified c-*kit* products performed. To distinguish the isoforms differing in 12 bp, amplification by PCR using the primers SRC-01 and 447 (refer to Table 2.5.2.1 for sequence locations) was carried out and the products resolved in a 4% Nusieve gel. As can be seen in Figure 5.4.4(A) the products possessing the 12 bp were 93 bp in size and those which lacked the 12 bp migrated slightly faster with a size of 81 bp. The 3 bp encoding the serine residue lay within the recognition site for the restriction endonuclease Pst I. Removal of the 3 bp altered the recognition site of Pst I which was therefore unable to cut the 792/780 bp (12+/12- included) c-*kit* PCR product generated by amplification with the primers 447 and 1004 (refer to Table 2.5.2.1 for sequence locations). However inclusion of the 3 bp resulted in the generation of the predicted 2 fragments of 658/646 bp (12+/12- included) and 134 bp in size (Figure 5.4.4 (B)).

5.5 *Transformation of epNIH/3T3 cells by the c-Kit isoforms*

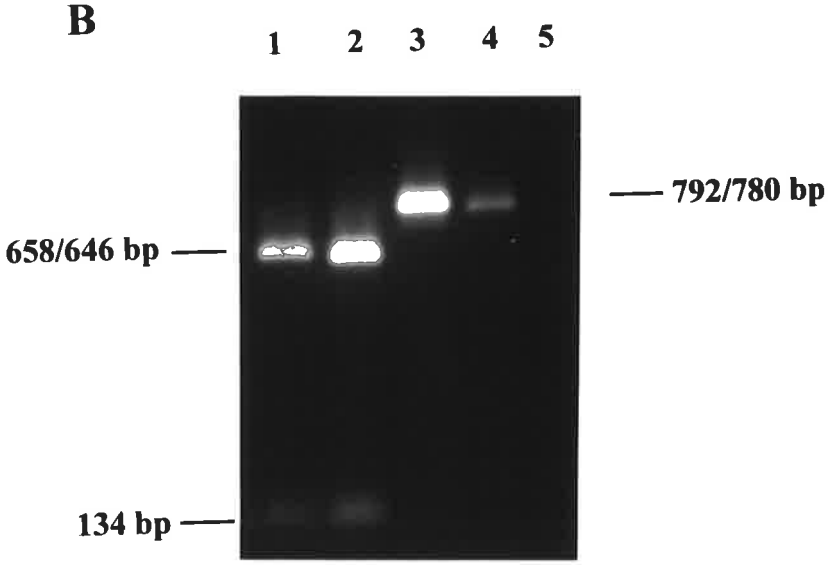
At subconfluency, in the absence or presence of 100 ng/ml hSLF epNIH/3T3 cells expressing the c-Kit(GNNK+S+), c-Kit(GNNK-S+) and c-Kit(GNNK+S-) isoforms did not show any distinct morphological changes. epNIH(GNNK-S-) cells were not included in any of the subsequent assays due to their lack of c-Kit protein expression. When the cells were allowed to become confluent, the former c-Kit isoforms induced focus-formation to varying degrees in the presence of 100 ng/ml hSLF. The production of foci in the absence of hSLF was possibly due to autocrine stimulation of the human c-Kit receptor by endogenously produced mSLF, since mSLF is able to cross-react with the human c-Kit receptor (for example refer to Lerner *et al.*, 1991). An increase in focus-formation appeared to result from an increase in c-Kit expression. The epNIH(GNNK-S+) cells displayed the greatest ability to produce foci, compared to epNIH(GNNK+S+/-) cells expressing comparable levels of receptors, in the absence and presence of hSLF, with a dramatic increase in production being seen with the cells expressing the highest receptor level, epNIH(GNNK-S+).2 (Figure 5.5.1

Figure 5.4.4: Verification of the deletion/insertions of the 12 bp and 3 bp encoding the GNNK and S amino acids, respectively, in epNIH(GNNK+/-S+/-) cells. First strand cDNA was reverse transcribed from 200 ng of poly A^+ selected mRNA, harvested from the epNIH(GNNK+/-S+/-) cells, using the protocol described by the manufacturers of the First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Cat. No. 27-9261-01). cDNAs from the cells were amplified with the human c-*kit* specific primer pairs 447 and SRC-01 (refer to Table 2.5.2.1 for sequence and locations) and the PCR products were size fractionated on a 4% Nusieve agarose gel for detection of the 12 bp deletion/insertion **(A).** For the detection of the 3 bp deletion/insertion, cDNAs were amplified with the human c-*kit* specific primer pair 447 and 1004 (refer to Table 2.5.2.1 for sequence and locations), the PCR product was digested with Pst I and size fractionated on a 1% agarose gel **(B).** *GAPDH* cDNA was also amplified from the cells to verify the production of cDNA **(C).** Lanes: Figure (A and C) 1. c-*kit(GNNK+S+),* 2. c-*kit(GNNK+S-),* 3. c-*kit(GNNK-S+),* 4. c-*kit(GNNK-S-),* 5. RUFMC1*neo.* Lanes: Figure (B) 1. c-*kit(GNNK+S+)*, 2. c-*kit(GNNK-S+),* 3. c-*kit(GNNK+S-),* 4. c-*kit(GNNK-S-)*, 5. RUFMC1*neo.*

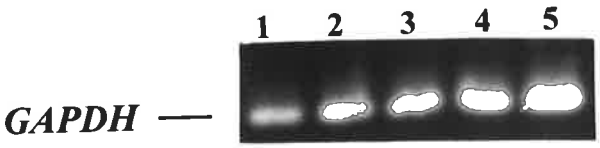
A



B



C



and Table 5.1). The latter also lost anchorage dependence in the presence of 100 ng/ml hSLF which became evident on day 10 of culture when the cells could no longer adhere to the substratum. As a consequence, dishes were also stained on day 8 compared to day 12 to visualise focus-formation of the epNIH(GNNK-S+).2 cells in the presence of ligand. Evidence of focus-formation by epNIH(GNNK+S+/-) cells on day 8 was minimal compared to that produced by epNIH(GNNK-S+) cells, therefore the results displayed in Table 5.1 and Figure 5.5.1 represent those obtained on day 12 except for epNIH(GNNK-S+).2 cells in the presence of hSLF which represent focus-formation on day 8. It should be noted that epNIH(GNNK-S+).10 and NIH(GNNK-S+).5 cells expressed similar levels of receptors and also produced similar levels of focus-formation as expected. epNIH(GNNK+S-) cells were slightly more effective in their ability to produce foci compared to epNIH(GNNK+S+) cells expressing similar levels of c-Kit expression (Table 5.1 and Figure 5.5.1). The foci produced by the epNIH(GNNK+S-) cells differed in morphology from those produced by the epNIH/3T3 infectants expressing the other isoforms, protruding more distinctly from the monolayer, with detachment of nearby cells from the substratum (Figure 5.5.1). In comparison, the pool of epNIH(mu*kit*) cells in the presence of 100 ng/ml mSLF did not produce distinct foci but the monolayers appeared more dense compared to those cultured in the absence of exogenous mSLF (Figure 5.5.1)(and as previously described in Chapter 4, Figure 4.2.4 and 4.8.3). The focus-formation results displayed in Table 5.1 and Figure 5.5.1 were confirmed in repeat experiments.

Examination of the saturation densities of the epNIH/3T3 cells expressing the different c-Kit isoforms demonstrated that at confluency only the cells expressing the c-Kit(GNNK-S+) isoform could reach higher cell densities than the RUFMC1*neo* control cells. An increase in c-Kit receptor levels in the epNIH(GNNK-S+) series of cells resulted in an increase in the saturation density obtained. Similar results were obtained in repeat experiments (refer to Table 5.1).

epNIH(GNNK+S+), epNIH(GNNK+S-) and epNIH(GNNK-S+) cells expressing various levels of c-Kit were plated in semi-solid agar to monitor their ability to grow in an anchorage independent manner. epNIH(GNNK+S+) and epNIH(GNNK-S+) cells were able to

Figure 5.5.1: Focus-formation of epNIH/3T3 infectants expressing the c-Kit isoforms. epNIH(Ruf*neo*), epNIH(GNNK+S+).10 and .2, epNIH(GNNK+S-).10 and .2, epNIH(GNNK-S+).10, .5 and .2 cells were cultured in the absence or presence of 100 ng/ml hSLF. epNIH(*neo*) and epNIH(mu*kit*) cells were cultured in the absence or presence of 100 ng/ml mSLF. Cultures were fed twice weekly for a period of 12 days. The confluent monolayers containing foci were stained using the Diff-Quick Kit (Lab-aids, Australia) and photographed. NIH(GNNK-S+).2 cells were also stained on day 8 due to the loss of contact of the cells to the substratum at day 10 .

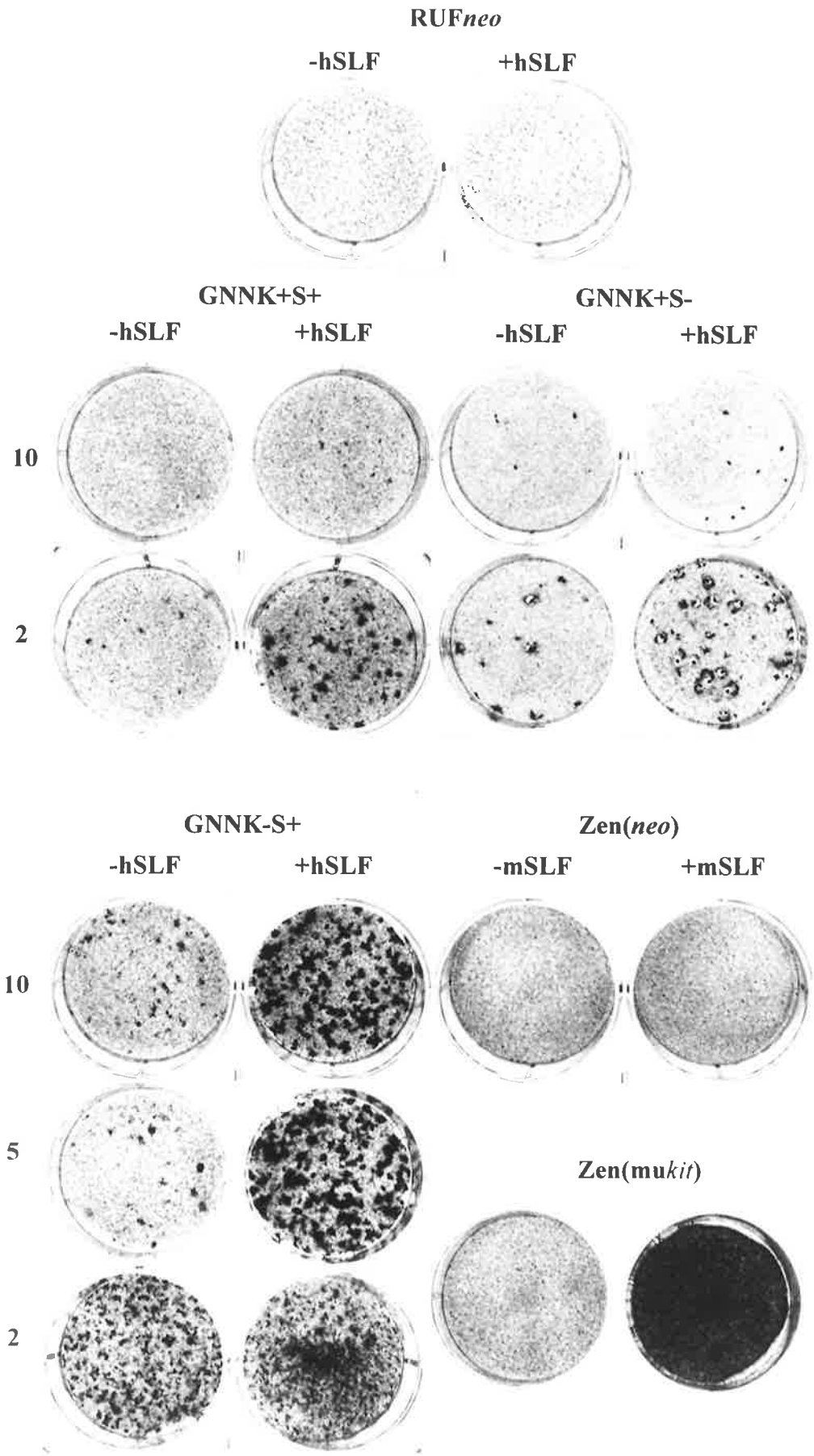

RUF*neo*
-hSLF
+hSLF
GNNK+S+
-hSLF
+hSLF
GNNK+S-
-hSLF
+hSLF
10
2
GNNK-S+
-hSLF
+hSLF
Zen(*neo*)
-mSLF
+mSLF
10
5
2
Zen(mu*kit*)

Table 5.1: Properties of the epNIH/3T3 cell lines expressing the c-Kit isoforms

epNIH/3T3 infectant	receptor no. x 10^3*	Colony Number$		Focus Formationδ		Saturation Densityξ $10^3/cm^2$	tumours/# of injections∞
		-SLF	+SLF	-SLF	+SLF		
RUFMC1*neo*	<1	7.25 ± 1.8	6.75 ± 1.5	0	0	47.50 ± 0.80	0/6
(GNNK+/Ser+).10	5.0	82.00 ± 5.7	97.75 ± 6.3	0	7	52.35 ± 2.26	0/6
(GNNK+/Ser+).5	10.8	40.50 ± 2.9	49.75 ± 2.2	nd	nd	51.93 ± 4.46	nd
(GNNK+/Ser+).2	17.9	17.25 ± 2.9	35.75 ± 5.4	7	49	54.33 ± 2.5	nd
(GNNK-/Ser+).10	2.9	57.70 ± 3.8(3)	77.50 ± 7.2	17	144	51.64 ± 4.6	nd
(GNNK-/Ser+).5	3.9	13.25 ± 2.3	17.75 ± 1.9	15	137	62.54 ± 4.8	4/6
(GNNK-/Ser+).2	19.5	7.00 ± 1.2	36.00 ± 4.0	115	>200	81.92 ± 3.9	nd
(GNNK+/Ser-).10	3.5	0.00 ± 0.0	0.00 ± 0.0	5	15	42.87 ± 5.0	0/6
(GNNK+/Ser-).2	6.7	0.00 ± 0.0	0.00 ± 0.0	9	39	44.29 ± 2.4	nd
Zen(*neo*)	nd	0.00 ± 0.0	0.00 ± 0.0	0	0	nd	0/6
Zen(mu*kit*)	nd	12.75 ± 1.9	204.30 ± 25.2	0	dense monolayer	nd	5/6

* Receptor numbers calculated using QIFIKIT beads as described in section 2.2.4. Receptor numbers/cell represented as the average of two experiments. Relative murine c-*kit* mRNA and c-Kit protein levels expressed by cells infected with the pZen(mu*kit*) construct were determined as described in section 5.7.

$ 10^4 cells plated in 0.33% soft agar in the absence or presence of 100 ng/ml hSLF except cells infected with pZen(*neo*) and pZen(mu*kit*) which were cultured in 100 ng/ml mSLF. Colonies comprising >50 cells were scored microscopically on day 14. Typical results of one of three repeated experiments are represented.

δ 5 x 10^4 cells seeded into 3.5 cm diameter wells. Cells were fed twice weekly in the absence or presence of 100 ng/ml hSLF or mSLF for cells infected with pZen(*neo*) and pZen(mu*kit*). Plates stained on day 12 except for epNIH(GNNK-S+).2 cells in the presence of hSLF due to loss of contact to the substratum on day 10 as a result the number of foci represented in the table for these cells in the presence of ligand were determined on day 8. Typical results of one of three repeated experiments are represented.

ξ 5 x 10^4 cells seeded into 6 cm diameter dishes and allowed to become confluent prior to trypsinisation and determining cell numbers and viability by haemocytometer counts and trypan blue exclusion.

∞ 8 week old female Balb/c *nude* mice were injected subcutaneously with 8 x 10^6 cells in the left and right hind flanks. Tumour production and size were monitored twice weekly. Tumours induced by the epNIH(GNNK-S+).5 cells were 5 mm in diameter (average) on day 42 and reached 10 mm in diameter (average) by day 55 at which stage the mice were sacrificed. epNIH/3T3 cells infected with the pZen(mu*kit*) construct (refer to Chapter 4) produced tumours (> 2 mm in diameter) with an average latency period of 50 days.

nd = not determined

grow in an anchorage independent manner in the absence and presence of 100 ng/ml hSLF. This was in contrast to epNIH(GNNK+S-).10 and .2 cells which reproducibly were unable to produce colonies even though they expressed similar receptor numbers to epNIH(GNNK-S+).5 and epNIH(GNNK+S+).10 cells, respectively (refer to Table 5.1). Interestingly, as had been demonstrated by the NIH(C3A3) series of cells, the epNIH(GNNK+/-S+) infectants expressing the lowest level of c-Kit surface protein had a greater ability to grow in an anchorage independent manner than those expressing higher levels. A 2 fold increase in receptor expression on epNIH(GNNK+S+).5 cells compared with epNIH(GNNK+S+).10 cells resulted in an approximately 2 fold decrease in colony number in the presence and absence of factor. Interestingly epNIH(GNNK-S+).10 and epNIH(GNNK-S+).5 cells, which expressed similar levels of receptors, produced a 4 fold difference in colony production which was in contrast to the similar levels of focus-formation produced by these two cell lines. These results were confirmed in repeat experiments.

As demonstrated in Chapter 4, colonies were also produced in the absence of exogenous factor (Table 5.1). This may be due to autocrine stimulation of the c-Kit receptor via murine SLF produced by the epNIH/3T3 cells as described above. However, only a 20-25% increase in colony number was seen in the presence of 100 ng/ml by the epNIH(GNNK+/-S+).10 and .5 cells and in the presence of 200 ng/ml no further increase in colony number was produced by epNIH(GNNK+/-S+).10 cells (data not shown). However, epNIH(GNNK+S+).2 cells expressing higher levels of surface c-Kit protein demonstrated a 50% increase in colony production in the presence of 100 ng/ml hSLF. epNIH(GNNK-S+).2 cells which expressed slightly higher receptor numbers, yet the most comparable to the epNIH(GNNK+S+).2 cells, also showed a greater increase in colony number in the presence of hSLF (~80%) in comparison to the lower expressing epNIH(GNNK-S+) cells. In the presence of hSLF epNIH(GNNK+S+).2 and epNIH(GNNK-S+).2 cells expressing comparable levels of c-Kit produced comparable levels of anchorage independent growth. However at the lower receptor levels epNIH(GNNK+S+).10 gave ~ 80% more colonies than epNIH(GNNK-S+).5 cells which expressed similar receptor levels. In comparison to the epNIH(mu*kit*) infectants in the absence of exogenous mSLF, the epNIH/3T3 infectants

expressing human c-Kit were generally more efficient at producing colonies. However, epNIH(mu*kit*) cells in the presence of mSLF were more efficient at producing colonies than the human c-Kit expressing cells in hSLF (Table 5.1).

5.6 *Tumourigenicity in nude mice*

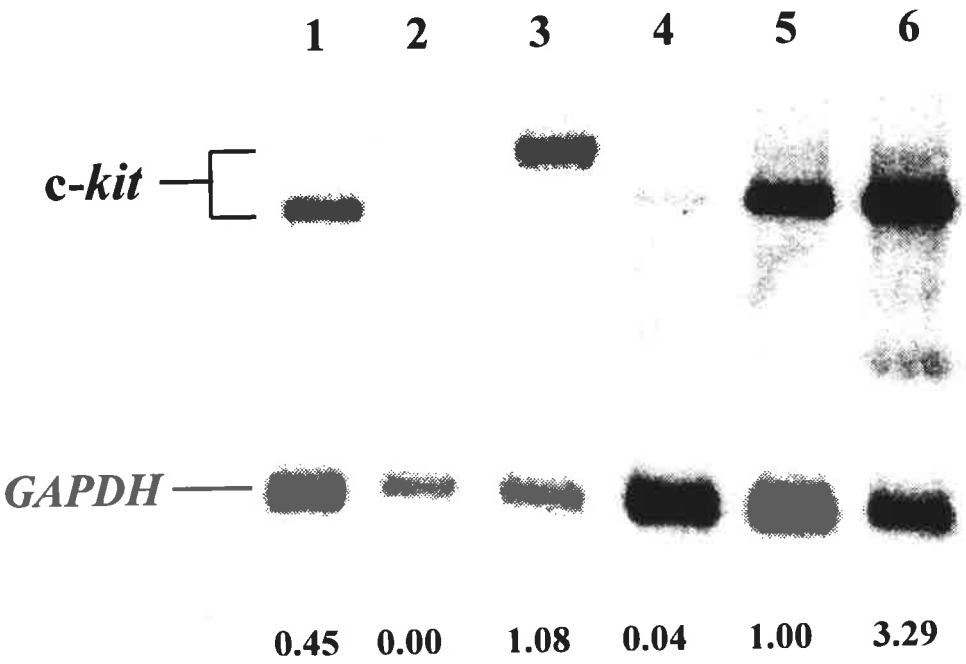
epNIH/3T3 infectants expressing the most comparable levels of c-Kit surface protein for each isoform were chosen to monitor their ability to induce tumours in *nude* mice. Only the epNIH/3T3 cells expressing the c-Kit(GNNK-S+) isoform were able to induce tumours *in vivo* with a latency period of approximately 42 days (refer to Table 5.1). As previously demonstrated in Chapter 4 epNIH(mu*kit*) cells also produced tumours.

5.7 *Comparative analysis of the levels of c-kit expression in NIH(mukit) and epNIH(GNNK+/-S+/-) cells*

To determine how the levels of c-*kit* in NIH(mu*kit*) (Chapter 4) compared with those expressed in the epNIH/3T3 cells expressing the human c-Kit isoforms northern blot analysis was performed. Murine and human c-*kit* transcripts were detected using a ^{32}P-labelled 63 bp DNA probe generated by PCR (kindly produced by Mr. Steve Fitter, Hanson Centre for Cancer Research, Adelaide, Australia) using the primers 1868 and 1869 (refer to Table 2.5.2.1 for sequence location). This probe had 100% homology to both the human and murine c-*kit* sequences (Yarden *et al.*, 1987a; Qui *et al.*, 1988). Due to the paradox between c-*kit* mRNA and c-Kit protein levels in the epNIH(GNNK+/-S+/-) cells a direct comparison between the c-*kit* mRNA levels expressed by NIH(mu*kit*) and epNIH(GNNK+/-S+/-) cells was not made as in the latter this is not reflective of the level of surface protein expressed by the cells. Since the NIH(mu*kit*) cells (Chapter 4) and the human leukaemic cell lines (Cole *et al.*, 1996) had demonstrated the expected correlation between c-*kit* RNA and c-Kit protein (ie as c-*kit* RNA increased so to did c-Kit protein) a comparison was made at the mRNA level between NIH(mu*kit*) cells and the human megakaryocytic cell line, M07e (Figure 5.7.1). Determination of the c-*kit/GAPDH* signal ratio of NIH(mu*kit*) cells demonstrated that they expressed comparable levels of c-*kit* mRNA to M07e cells which have approximately

Figure 5.7.1: Level of c-kit expression in a panel of murine and human c-kit expressing cell lines. Poly A^+ selected mRNA (2μg) was analysed by agarose gel electrophoresis and northern blot hybridisation. Murine and human c-*kit* signals were detected using a ^{32}P-labelled 63 bp probe which had 100% homology to both species of c-*kit*. The membrane was also probed with a 780 bp human GAPDH fragment. Signals were detected, visualised and digitised using a Molecular Dynamics PhosphorImager. Hybridisation signals were quantified and represented as c-*kit/GAPDH* ratios relative to the M07e signal. The size of the murine

c-*kit* transcript detected in P815 (lane 1) was 5.2 kb, no transcript was detected in NIH(*neo*) cells (lane 2), a 7 kb transcript was detected in the NIH(mu*kit*) cells which had been infected with the pZen(mu*kit*) construct (refer to Chapter 4) (lane 3), and a 5.2 kb human c-kit transcript was detected in HEL (lane 4), M07e (lane 5) and HMC-1 (lane 6) cells.


1
2
3
4
5
6
c-kit
GAPDH
0.45
0.00
1.08
0.04
1.00
3.29

3.6 x 10^5 receptors/cell (Cole *et al.*, 1996). Based on this comparison it can crudely be estimated that NIH(mu*kit*) cells express in the order of 20 fold higher levels of surface protein compared to epNIH(GNNK+/-S+).2 cells (average 1.9 x 10^4 receptors/cell; Table 5.1).

5.8 DISCUSSION

The functions of the naturally occurring human c-Kit isoforms (GNNK+/-S+/-) as yet are not fully understood. Several studies have analysed the expression of these isoforms at the RNA level and shown that transcripts for the alternative splice variants are co-expressed in normal and leukaemic cells (Crosier *et al.*, 1993; Furitsu *et al.*, 1993; Piao *et al.*, 1994). However to our knowledge there have not been any published data directly comparing the biological function of cells independently expressing the different human c-Kit isoforms. This chapter therefore addressed whether the human c-Kit isoforms displayed any discernible differences in their ability to transform the indicator epNIH/3T3 cell line.

Introduction of the cDNAs encoding the c-Kit isoforms into epNIH/3T3 cells resulted in expression of c-*kit* mRNA transcripts of the expected sizes for the four variants. Upon analysis of c-Kit surface protein expression all isoforms except c-Kit(GNNK-S-) could be detected. Lack of intracellular protein by the APAAP technique in the latter cells excluded the possibility that c-Kit(GNNK-S-) was being proteolytically cleaved, removing it from the surface, as is thought to occur in the production of the soluble c-Kit receptor (Yee *et al.*, 1993, 1994a; Turner *et al.*, 1995). It is surprising that c-Kit(GNNK-S-) protein could not be expressed since the murine cDNA used in Chapter 4 was equivalent to this isoform. It is possible that the combination of the two deletions (12 bp and 3 bp) may affect translational events or the turnover/degradation of the human c-Kit receptor. Concerning the latter it has recently been demonstrated that a specific mutation in the intracellular domains of both the c-Kit and CSF-1 receptors accelerates the rate of degradation of these receptors resulting in reduced levels of protein (Kitayama *et al.*, 1995; Glover *et al.*, 1995). A similar mechanism may explain the results obtained with the c-Kit(GNNK-S-) isoform resulting in levels of protein too low to be detected by the methods employed in this study, even though cells expressed c-*kit* mRNA. Due to the lack of protein in epNIH(GNNK-S-) cells they were not

included in the transformation assays, however in light of the recent data described by Glover *et al.*, (1995) the ability of epNIH/3T3 cells expressing c-*kit(GNNK-S-)* mRNA to undergo transformation should be assessed in the future.

The c-Kit isoforms that could be detected at both the mRNA and protein levels, all demonstrated the ability to induce at least one of the phenotypic characteristics of transformation in epNIH/3T3 cells. c-Kit(GNNK-S+) induced the most potent transformation enabling cells to grow to higher densities, to produce foci and anchorage independent colonies *in vitro* and tumours in *nude* mice. epNIH(GNNK+S+) cells were able to produce foci and anchorage independent growth but did not induce tumours, while c-Kit(GNNK+S-) could only induce focus-formation (refer to Table 5.1). Not only did this demonstrate that the c-Kit isoforms displayed distinctive transforming capabilities but that the three assays were detecting potentially independent functional aspects of the different isoforms.

The more potent transforming capability of c-Kit(GNNK-S+) compared to the c-Kit(GNNK+) isoform expressed at comparable levels (summarised in Table 5.1) may be reflective of the enhanced activation demonstrated by c-Kit(GNNK-) receptor in the mouse (Reith *et al.*, 1991; Blouin and Bernstein, unpublished). In particular, murine c-Kit(GNNK-) displayed a significant level of constitutive phosphorylation in contrast to c-Kit(GNNK+). Analysis of the degree of tyrosine phosphorylation of human c-Kit(GNNK-) compared to c-Kit(GNNK+) in the absence of hSLF needs to be performed to determine whether the greater transforming ability of the human c-Kit(GNNK-) isoform can be attributed to this.

The effect of the deletion of the serine residue was evident from the inability of the c-Kit(GNNK+S-) isoform to induce anchorage independent growth, although it did not affect focus-formation. It has recently been demonstrated that anchorage independent growth and focus-formation induced by a CSF-1/αPDGF hybrid receptor can be dissociated (Yu *et al.*, 1995). The binding of PLC-γ1 to the αPDGF intracellular portion of the hybrid receptor was essential for efficient focus-formation but not required for anchorage independent growth, therefore demonstrating that these attributes of transformation are mediated by different signalling pathways. An increasing number of signalling molecules have been shown to associate with c-Kit and it may be that S_{715} is involved in the binding of one of these which

in turn may be essential for anchorage independent growth induced by the human c-Kit receptor. A possible candidate may be p85, the regulatory subunit of PI 3'-K, which has been postulated to bind to Y_{721} (the homologue of Y_{719} in the murine c-Kit receptor shown to be involved in the binding of p85) (Shearman *et al* 1993, Serve *et al.*, 1994) which lies in close proximity to S_{715}. It is possible that the loss of S_{715} could affect the binding of p85 which may be essential for anchorage independent growth but not required for focus-formation induced by the human c-Kit receptor. While at first sight this seemed an attractive possibility it has been shown that p85 binding to the CSF-1R and αPDGFR, which have high homology with the c-Kit receptor, is not required for anchorage independent growth (Taylor *et al.*, 1989; Yu *et al.*, 1995). However, these receptor tyrosine kinases have diverse biological functions due to variation in their substrate specificities and therefore the binding of p85 to the c-Kit(S+/-) isoforms is currently being examined in the laboratory to investigate this issue.

As for the murine c-Kit receptor in chapter 4, the effect of receptor density on cell transformation was also analysed for the human c-Kit isoforms. Cells expressing increasing levels of human c-Kit receptors were obtained using two independent methods. NIH/3T3 cells transfected with the pGC1.2 vector containing the c-*kit(GNNK+S+)* cDNA as well as the *DHFR* gene allowed for the selection of the NIH(C3A3) series of cells which expressed progressively increasing levels of c-Kit(GNNK+S+) by culturing in increasing concentrations of Mtx. epNIH/3T3 cells expressing various levels of the different c-Kit isoforms were also obtained by infection with the pRUF(*GNNK+/-S+/-*) constructs and fluorescence-activated cell sorting. In both systems an inverse relationship was generally seen between the levels of c-*kit* mRNA and protein expression, whereas in the previous chapter murine c-*kit* mRNA and protein levels showed the expected tight positive correlation. The paradox between mRNA and protein levels did not appear to be a consequence of the pRUF(*GNNK+/-S+/-*) constructs since factor-dependent cell lines also infected with these (described in Chapter 6) demonstrated the expected positive correlation between c-Kit protein and c-*kit* mRNA levels as did a series of c-Kit expressing leukaemic cell lines (Cole *et al.*, 1996). The inverse relationship seen in the (ep)NIH/3T3 cells is not understood at present and will require further analysis for this to be resolved. It should be noted however, that a similar inverse relationship

between RNA and surface protein levels has also been reported for the T cell receptor during T cell maturation (Magurie *et al.*, 1990).

Since the levels of c-Kit protein increased as expected in the NIH(C3A3) Mtx selected cell lines, expressing c-Kit(GNNK+S+), and the epNIH(GNNK+/-S+/-) sorted populations this was subsequently used to compare c-Kit expression levels between cells. However, this is also an approximate estimation of the level of c-Kit protein surface expression due to the possibility of SLF-mediated internalisation of the human receptor as a result of the cross-species reactivity of murine SLF (Lerner *et al.*, 1991) produced by the cells. It is also possible that the extent of ligand-induced down-modulation of the different c-Kit isoforms may also vary, which will be addressed in more detail in Chapter 6. With these reservations, the surface protein levels expressed by the epNIH(GNNK+/-S+/-) infectants were all lower than that expressed on the human erythroid leukaemic cell line, HEL (~2.6 x 10^4 receptors/cell). Using the QIFIKIT® method with HEL as a standard, comparisons could be made between the relative number of c-Kit receptors expressed by the epNIH/3T3 infectants and a panel of leukaemic cell lines and normal $CD34^+$ fractionated bone marrow cells as determined in a recent study in the laboratory using the same method of quantitation (Cole *et al.*, 1996; Table 1). The majority of the epNIH/3T3 c-Kit infectants expressed less receptors on their surface than the low c-Kit expressing leukaemic cell line RC-2A (7.8 x 10^3 receptors/cell) and c-Kit receptor levels on the NIH(GNNK+/-S+).2 (average 1.9 x 10^4 receptors/cell) were comparable to those expressed on normal $CD34^+$ bone marrow cells (1.96 x 10^4 ± 1.31 receptors/cell). Transformation of epNIH/3T3 cells could therefore be induced by levels of c-Kit comparable to physiological levels. However as shown in Chapter 4 higher than physiological levels of murine c-*kit* were required to induce transformation. It was roughly estimated in this chapter that NIH(mu*kit*) cells expressed approximately 20 fold higher levels of c-Kit expression compared to NIH(GNNK+/-S+).2 cells based on comparisons made at the c-*kit* mRNA level between NIH(mu*kit*) and M07e cells (described in more detail in section 5.7). We acknowledge that this is only a rough estimate as several parameters may differentially influence the final level of c-Kit protein expressed on the surface of NIH(mu*kit*) and M07e cells. These may include intracellular

interactions between c-Kit and SLF preventing a proportion of the c-Kit protein translated from reaching the surface and also SLF-mediated internalisation of the receptor which would influence the levels of protein detected on the surface of NIH(mu*kit*) cells compared to M07e cells. Scatchard analysis commonly used to determine receptor levels would certainly expand on the results described in this chapter. However it must be pointed out that the limitations described above will also influence the results by that method. Thus it was demonstrated that higher levels of murine compared to human c-Kit were required to induce transformation and that overexpression of human c-Kit relative to physiological levels was not required for transformation.

Comparisons made between cells expressing different human c-Kit receptor densities demonstrated that an increase in expression resulted in an increase in factor-dependent and independent focus-formation for all the isoforms analysed. An increase in c-Kit(GNNK-) expression further reflected the enhanced transforming ability of this isoform as it was able to produce more foci and grow to higher densities compared to cells expressing comparable levels of the c-Kit(GNNK+) isoforms. Surprisingly, anchorage independent growth decreased with respect to an increase in human c-Kit receptor density. The latter was demonstrated by both the epNIH(GNNK+/-S+/-) infectants and also the NIH(C3A3) series of cells, therefore eliminating the original suggestion that reduction in colony number in the NIH(C3A3) series was a consequence of the affects of high MTX concentrations. The progressive increase in c-Kit surface protein expression by the NIH(C3A3) series demonstrated that a "window" of c-Kit surface protein expression may be required to achieve maximal anchorage independence. Low c-Kit receptor levels expressed by the unselected NIH(C3A3) cells (~1 x 10^3 receptors/cell also determined using the QIFIKIT® method) did not lead to production of colonies and nor did the highest c-Kit receptor levels obtained in this study (ie cells selected in > 400 nM Mtx, expressing > 8 x 10^3 receptors/cell). It appeared that NIH(C3A3)$_{200\ nM}$ cells expressing intermediate levels of c-Kit (~ 4 x 10^3 receptors/cell) comparable to those expressed by NIH(GNNK+S+).10 cells (Table 5.1) were sufficient to induce maximal colony production in the NIH(C3A3) Mtx resistant cell lines. Although not addressed in this study the "window" of expression necessary for maximal anchorage independent growth may be a

consequence of the degree of ligand-mediated receptor tyrosine kinase activation achieved at varying receptor densities. There may be a threshold level of receptor tyrosine kinase activation required prior to the induction of transformation. Higher receptor levels may result in extremely high levels of tyrosine kinase activation, either induced by ligand or possibly as a result of spontaneous dimerization, which would greatly amplify the recruitment and phosphorylation of cytoplasmic molecules involved in signal transduction. A disruption in the balance of the signalling cascade of events may lead to inhibition rather than induction of anchorage independent growth. This may be analogous to the decrease in proliferation seen upon stimulation of cells overexpressing the EGF or HER-2/*neu* receptors (as previously discussed in Chapter 4) which was associated with high levels of tyrosine kinase activity (Kawamoto *et al.*, 1984; Riedel *et al.*, 1987; Lupu *et al.*, 1990). The decrease in anchorage independent growth was also demonstrated by the epNIH(GNNK+/-S+) cells as the level of c-Kit receptors increased and similar events may also explain why the NIH(mu*kit*) clones R56 and R62, expressing the highest levels of murine c-Kit in chapter 4, produced reduced numbers of colonies. It should be noted that the number of colonies produced in the anchorage independence assay by cells expressing c-Kit(GNNK-) were not higher than those produced by c-Kit(GNNK+) cells expressing comparable levels but in fact were lower. Thus this may further reflect that enhanced tyrosine kinase activation could inhibit anchorage independent growth. This phenomenon was not displayed in the focus-formation assay again suggesting that these two attributes of transformation can be dissociated. Analysis of the degree of tyrosine kinase activation of these c-Kit receptors expressed at varying densities and the subsequent association with signalling molecules may aid in explaining the results described above.

In the absence of recombinant SLF, NIH(GNNK+/-S+) cells produced anchorage independent growth as had been described in the previous chapter for NIH(mu*kit*) cells. In the case of the human c-Kit isoforms hSLF-independent transformation may also be partially attributed to autocrine stimulation by endogenously produced murine SLF due to the cross-species reactivity of murine SLF (Lerner *et al.*, 1991). However, the frequency of hSLF-independent colony formation obtained with the human c-Kit isoforms at the low

receptor levels was much higher than that obtained with the murine c-Kit receptor even though the level of c-Kit expression in the epNIH(mu*kit*) cells is higher as described above (although estimates were determined using NIH(mu*kit*) cells it was shown in chapter 4 that epNIH(mu*kit*) cells expressed equivalent levels of c-*kit* mRNA and protein). Given that murine SLF has a lower affinity for human c-Kit than for murine c-Kit it is hard to attribute the higher frequency to autocrine stimulation alone. Thus it is possible that spontaneous dimerization may be contributing to the results obtained in the absence of hSLF. Interestingly, the degree of anchorage independent colony formation in the presence of hSLF generally displayed much less enhancement (16 - 26%) compared to cells expressing the highest receptor levels NIH(GNNK+S+/-).2 (50%) and the epNIH(mu*kit*) pool of cells in the presence of mSLF (~90%) (Table 5.1). At present the results obtained by the anchorage independent assay are not fully understood. Future analysis involving the kinetics of tyrosine kinase activation, receptor turnover, dimerization and whether the binding kinetics of hSLF are affected in the presence of endogenous murine SLF may aid in explaining the results demonstrated in this Chapter.

CHAPTER 6

Responsiveness of factor-dependent cell lines expressing the human c-Kit isoforms to soluble SLF.

6.1 INTRODUCTION

The c-*kit* proto-oncogene product is a growth factor receptor normally expressed on early haemopoietic progenitor cells and mast cells which are dependent on SLF alone or in combination with other cytokines for their survival, proliferation and differentiation (Ashman *et al.*, 1991; Simmons *et al.*, 1994; Valent *et al.*, 1989; Irani *et al.*, 1992; Nilsson *et al.*, 1993). It therefore seemed appropriate to evaluate the c-Kit(GNNK+/-S+/-) isoforms in haemopoietic cells. Furthermore, NIH/3T3 cells, used in the previous chapter, did not provide an appropriate background to analyse the potential differential responsiveness of the c-Kit isoforms to human SLF (hSLF), due to interference of autocrine stimulation by murine SLF. Therefore cDNAs encoding the c-Kit(GNNK+/-S+/-) were introduced into the murine early myeloid, factor-dependent FDC-P1 and 32D cell lines. Preliminary studies were undertaken to investigate the responsiveness of the various isoforms to SLF.

Expression of the human c-Kit isoforms in factor-dependent cell lines would also enable us to address whether hSLF-driven proliferation of the infectants could be differentially inhibited by a panel of anti (α)-c-Kit monoclonal antibodies (mAb). The mAb used in this study included the well characterised SR-1 (Broudy *et al.*, 1992), 17F11 (Bühring *et al.*, 1991) and YB5.B8 (Gadd & Ashman, 1985; Lerner *et al.*, 1991) which bind to different epitopes of the extracellular domain of the c-Kit receptor (Ashman *et al.*, 1994) and a less characterised mAb, 1DC3 (Aylett *et al.*, 1995). The rationale behind these studies was based on several reports describing that YB5.B8 could inhibit a number of SLF-dependent processes such as the production of granulocyte/macrophage colony forming units (CFU-GM) (Cambareri *et al.*, 1988; Ashman *et al* 1990), stroma-dependent CFU generation by $CD34^+$ cord blood cells (Cicuttini *et al.*, 1992), haemopoietic cell adhesion to stroma (A. Zannettino; unpublished data), SLF-dependent mast cell differentiation (Valent *et al.*, 1992) and natural killer (NK) cell proliferation (Matos *et al.*, 1993). However, in a recent study it was shown that YB5.B8 had no inhibitory effect on the hSLF-driven proliferation of the human megakaryocytic, M07e and erythroleukaemic, TF-1 cell lines (Ashman *et al.*, 1994). Since these cells have been shown to co-express the c-Kit(GNNK+/-S+/-) isoforms, with a predominance of GNNK- and Ser+ containing variants (Crosier *et al.*, 1993), it was possible

that differences in the ratios of the c-Kit isoforms contributed to the variable inhibition seen by YB5.B8. This chapter therefore investigates whether YB5.B8 and the other mAb described above differ in their abilities to block the hSLF-driven proliferation of the factor-dependent cell lines independently expressing the different human c-Kit isoforms.

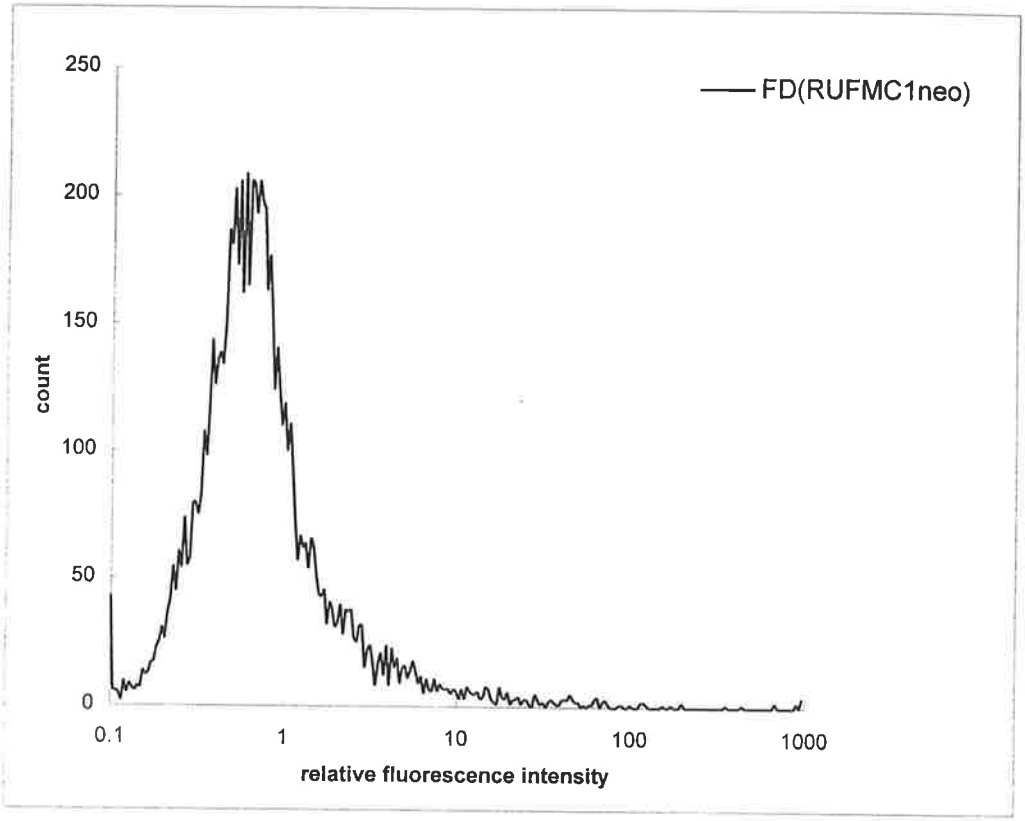
RESULTS

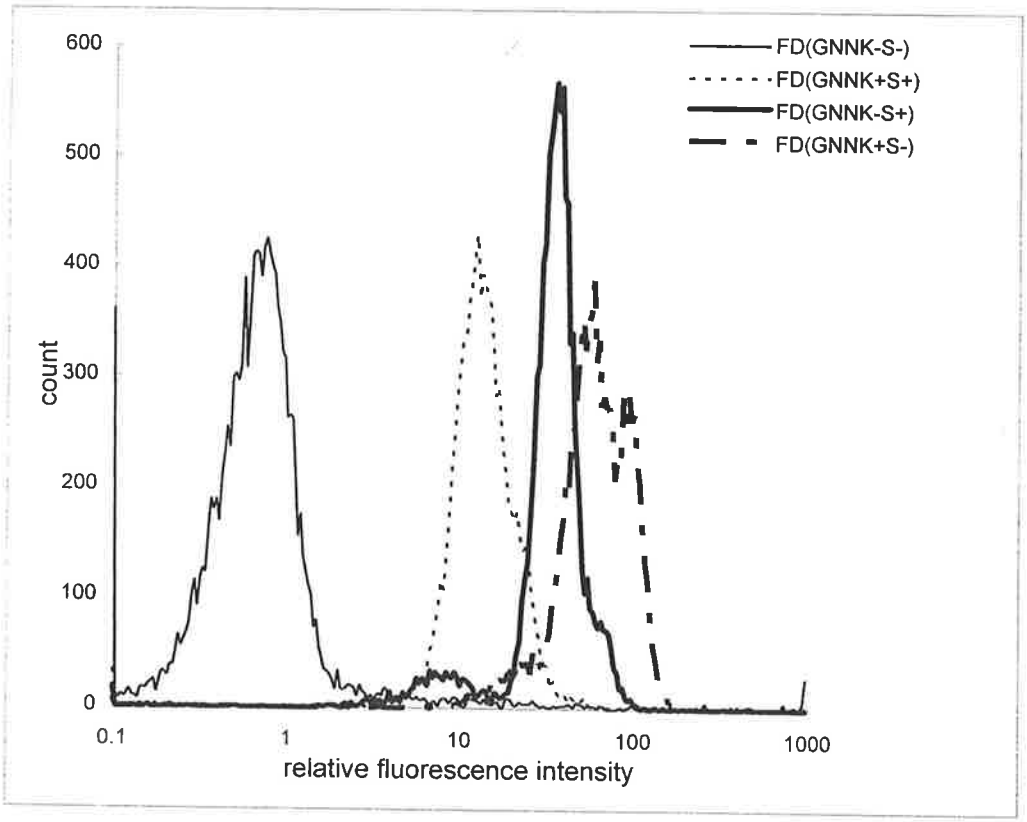
6.2 *Introduction of the c-KIT isoforms into the factor-dependent cell line FDC-P1.*

FDC-P1(SLF) cells which had been selected to grow in murine SLF (mSLF), as described in Chapter 3, were infected by co-culture with the ψ2 packaging cell lines producing pRUF(GNNK+/-S+/-) viruses (refer to section 2.8.5 for method). These cells were chosen for infection as they were known to contain the machinery required for responses initiated by the endogenous murine c-Kit receptor. Co-culture experiments were performed in the presence of residual levels (0.5 U/ml) of murine GM-CSF (mGM-CSF) and 40 ng/ml hSLF for 3 days, prior to G418 selection. This combination of growth factors was chosen as it had been shown in preliminary studies that infection in the presence of hSLF alone or in a combination of mGM-CSF/mSLF did not produce infectants that expressed human c-Kit on their surface (data not shown). After G418 selection the mGM-CSF was gradually eliminated from the culture medium and the level of hSLF increased to 50-100 ng/ml. Control cells were infected with RUFMC1*neo* retrovirus not containing the c-*kit* insert and co-culture performed in mGM-CSF. FD(GNNK+S+), FD(GNNK-S+), FD(GNNK+S-) and FD(GNNK-S-) cells able to proliferate/survive in cell culture in hSLF alone were obtained and were routinely maintained in 50 ng/ml of hSLF. Since these cells were maintained in hSLF, determination of the levels of surface c-Kit protein would be influenced by SLF-dependent internalisation of the receptor. Therefore cells were washed to remove residual hSLF and cultured in mGM-CSF overnight prior to assaying for surface c-Kit protein expression by indirect immunofluorescence (IIF) using the α-c-Kit mAb, 1DC3 (Figure 6.2.1). This revealed that the levels of expression of the c-Kit isoforms varied in the following order: c-Kit(GNNK+S-) > c-Kit(GNNK-S+) > c-Kit(GNNK+S+). c-Kit(GNNK-S-) again could not be detected on the surface of the cells yet they were able to survive in hSLF alone, but did not proliferate in a

Figure 6.2.1: Expression of c-Kit surface protein on FD(GNNK+/-S+/-) cells.

FD(GNNK+/-S+/-) cells maintained in hSLF and control FD(RUFMC1*neo*) cells maintained in mGM-CSF were washed and cultured in 40 U/ml mGM-CSF overnight. Cells were labelled with the α-c-Kit mAb 1DC3 and binding detected with a second stage PE-labelled α-Ig reagent and flow cytometric analysis.


FD(RUFMC1neo)
250
200
150
100
50
0
count
0.1
1
10
100
1000
relative fluorescence intensity


FD(GNNK-S-)
FD(GNNK+S+)
FD(GNNK-S+)
FD(GNNK+S-)
600
500
400
300
200
100
0
count
0.1
1
10
100
1000
relative fluorescence intensity

hSLF-dependent manner (demonstrated in Figure 6.3.1 below). This suggested the possibility that FD(GNNK-S-) cells were factor-independent. To investigate this further all of the FD(GNNK+/-S+/-) cells were cultured in the absence of factor. FD(GNNK-S+) and FD(GNNK+S-) cells did not survive under these culture conditions. However, in the cultures initiated with FD(GNNK-S-) and FD(GNNK+S+) cells a population of cells arose which were factor-independent (FID). Analysis of surface c-Kit protein expression revealed that FID(GNNK+S+) cells retained c-Kit expression even after several weeks of culture in the absence of hSLF and proliferated just as well as cultures receiving factor (data not shown). FID(GNNK-S-) cells as expected were negative for c-Kit surface protein. Whether FID(GNNK+S+) cells had become factor-independent due to an event related to c-Kit was not analysed in this study. However the lack of c-Kit expression by FID(GNNK-S-) cells suggested they had been transformed by an event unrelated to c-Kit.

Northern blot analysis demonstrated that c-*kit* mRNA was also undetectable in FD(GNNK-S-) cells, but transcripts of the expected sizes (6.3 and 5.1 kb) were detectable in all other FDC-P1 c-Kit infectants (Figure 6.2.2). The *c-kit/GAPDH* ratios for each infectant correlated with the level of c-Kit protein seen in Figure 6.2.1 (for example FD(GNNK+S-) cells expressed the highest level of surface c-Kit protein and mRNA). Reverse transcribed cDNA obtained from the FDC-P1 infectants was analysed to verify that the correct isoforms were being expressed by the cells. This was performed by PCR amplification of c-*kit*, and subsequent size determination and restriction endonuclease digestion of the products, as was described in section 5.4 for the NIH(GNNK+/-S+/-) cells. All the FDC-P1 infectants expressed the correct isoforms, including FD(GNNK-S-) even though c-*kit* mRNA was undetectable by Northern blot analysis (data identical to that portrayed in Figure 5.4.4).

In order to compare the responsiveness of the c-Kit isoforms to SLF, cells expressing comparable levels of c-Kit surface protein were required and were obtained by IIF-activated cell sorting. Table 6.2.1. shows the receptor numbers expressed by the final cell populations collected after several rounds of cell sorting, determined by quantitative IIF using commercially available QIFIKIT® beads as described in section 2.2.4. FD(GNNK+S+) and FD(GNNK-S+) expressed similar levels of c-Kit with approximately 6.5 x 10^4 receptors/cell

Figure 6.2.2: Expression of c-*kit* mRNA in the FD(GNNK+/-S+/-) cells. Poly A^+ selected mRNA (2μg) collected from FD(GNNK+/-S+/-) cells cultured in hSLF and FD(RUFMC1*neo*) cells cultured in mGM-CSF were analysed by agarose gel electrophoresis and northern blot hybridisation. Hybridisation was performed using a full length human c-*kit(GNNK+S+)* probe and a 780 bp human *GAPDH* fragment labelled with ^{32}P. C-*kit* signals were quantitated by PhosphorImager analysis using *GAPDH* signals as a control for comparative loading. Transcripts obtained from the RUFMC1*neo* vector containing the c-*kit* sequences are indicated. Lanes 1: FD(GNNK+S+); 2. FD(GNNK+S-); 3. FD(GNNK-S+); 4. FD(GNNK-S-); and 5. FD(RUFMC1*neo*).

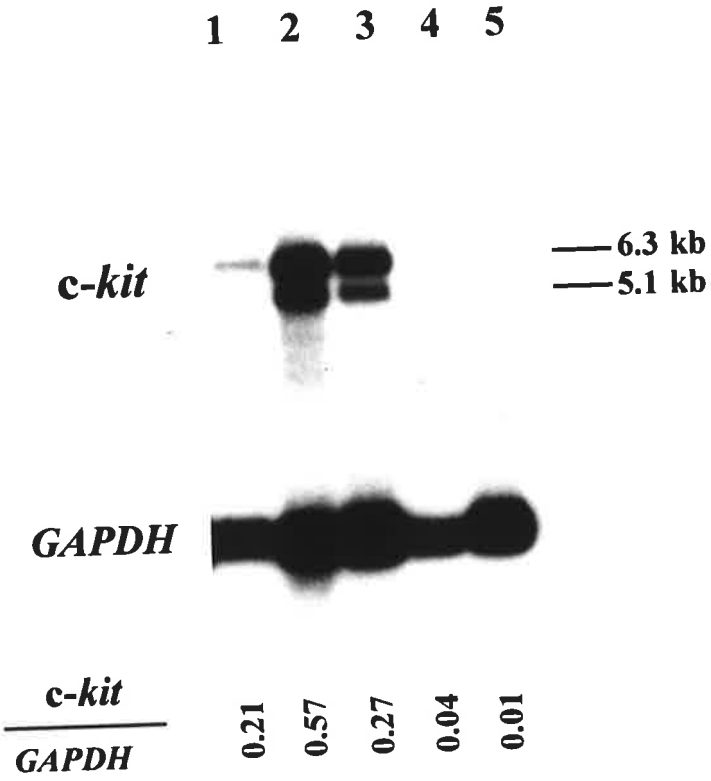

1 2 3 4 5
c-kit
6.3 kb
5.1 kb
GAPDH
c-kit / GAPDH
0.21
0.57
0.27
0.04
0.01

Table 6.2.1: Levels of c-Kit surface protein expression on cells cultured in the presence of hSLF or mGM-CSF

Cells	*receptors/cell (x 10^3)	mean fluorescence intensity#	
		hSLF	mGM-CSF
FD(GNNK+S+)	61 ± 1	11.6	50.7
FD(GNNK-S+)	68 ± 2	6.0	54.5
FD(GNNK+S-)	129 ± 10	18.1	72.3
FD(GNNK-S-)	<1	0	0
FD(RUFMC1*neo*)	<1	0	0
HEL	23 ± 3	nd	nd

*Cells were cultured overnight in mGM-CSF (40 U/ml) and receptor numbers quantitated using QIFIKIT® beads (see section 2.2.4 for method). Data represented as the mean of three individual experiments ± S.E.M.

#mean fluorescence intensities of cells cultured in 50 ng/ml of hSLF or 40 U/ml mGM-CSF overnight. Data represents typical results obtained from one of three repeat experiments.

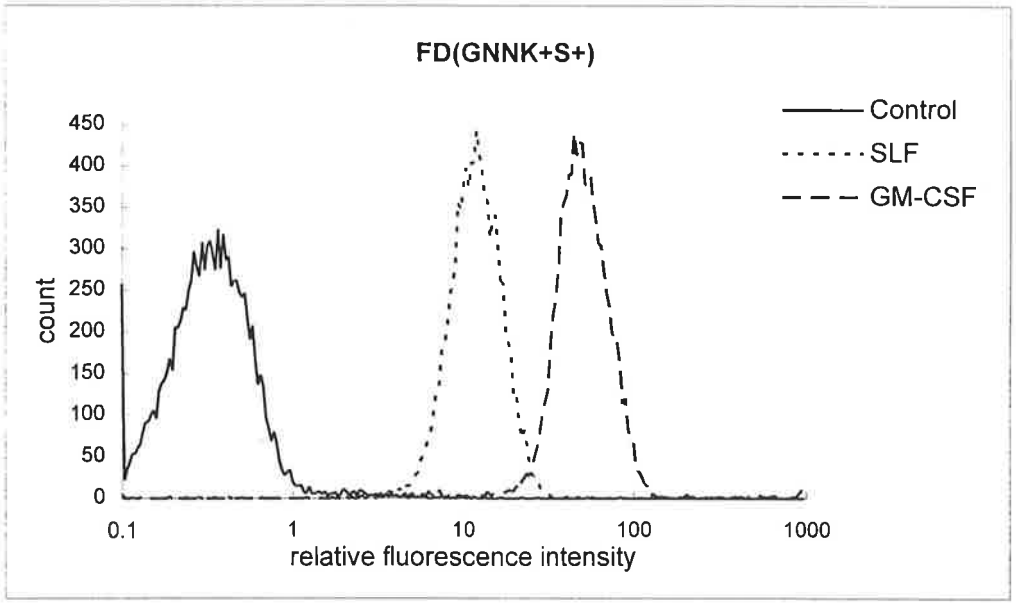
(average), however FD(GNNK+S-) cells expressed approximately 2 fold higher receptors/cell (~$1.3x10^5$). Several attempts were made to obtain more comparable levels of c-Kit expression between FD(GNNK+/-S+) and FD(GNNK+S-) cells with little success, therefore subsequent experiments were performed with the cell populations as described.

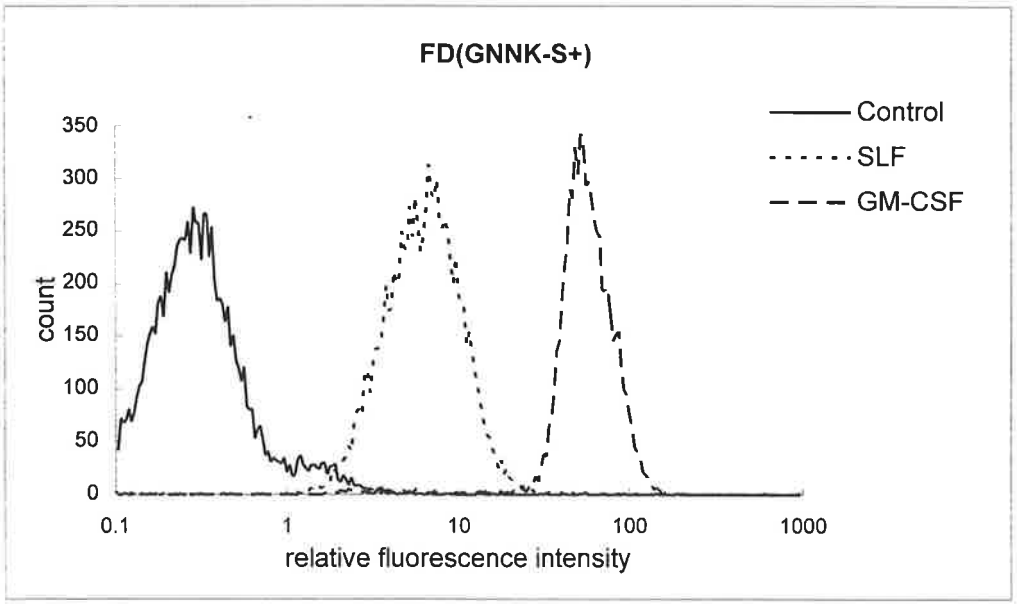
Comparisons of the levels of surface c-Kit protein on cells cultured overnight in the presence of mGM-CSF versus hSLF demonstrated that the FD(GNNK+S+/-) cells expressed ~ 4.3-fold lower levels of c-Kit protein when cultured in hSLF. FD(GNNK-S+) cells showed an even greater (9-fold) decrease in detectable c-Kit surface protein when cultured in hSLF. Since FD(GNNK-S+) cells expressed comparable receptor numbers to the FD(GNNK+S+) cells in mGM-CSF (Figure 6.2.3 and table 6.2.1), this suggested that the rate of SLF-mediated internalisation was increased by the deletion of GNNK. Another possibility was that the binding of hSLF to the c-Kit(GNNK-S+) receptor may be disrupting the 1DC3 mAb from binding to its epitope, possibly due to a differential conformational change induced by GNNK- compared to GNNK+. The latter possibility will be discussed further in section 6.5 below.

6.3 *Analysis of the responsiveness of the FDC-P1 cells expressing the different c-Kit isoforms to SLF*

Cells maintained in hSLF were washed prior to culture for 3 days with various concentrations of hSLF and proliferation monitored by ^{3}H-thymidine uptake (refer to section 2.3.1 for method). Cells expressing each isoform except c-Kit(GNNK-S-) displayed hSLF-dependent proliferation. FD(GNNK-S+) displayed the highest rate of ^{3}H-thymidine uptake of all the isoform expressing cell lines displaying an ~ 2.3-fold (average estimated throughout assay) higher proliferative capacity than FD(GNNK+S+) cells expressing comparable levels of c-Kit receptors. FD(GNNK+S-) cells achieved 1.5-fold (average) higher levels of proliferation than FD(GNNK+S+) cells over the range of SLF concentrations assayed which may be reflective of the 2-fold difference in their receptor numbers. M07e cells displayed the greatest proliferative capacity, except at 200 ng/ml at which FD(GNNK-S+) had reached approximately equivalent levels of proliferation, with FD(GNNK+S-/+) proliferating

Figure 6.2.3: Expression of c-Kit surface protein on FD(GNNK+/-S+/-) cells cultured in hSLF and mGM-CSF. Cells cultured in 50 ng/ml hSLF (·····) or 40 U/ml mGM-CSF (_ _ _) and labelled with the α-c-Kit mAb 1DC3 or an isotype-matched negative control mAb 3D3 (——). Binding of mAb to the cells was detected using a second stage PE-labelled α-Ig reagent and flow cytometric analysis.


FD(GNNK+S+)
Control
SLF
GM-CSF
count
450
400
350
300
250
200
150
100
50
0
0.1
1
10
100
1000
relative fluorescence intensity


FD(GNNK-S+)
Control
SLF
GM-CSF
count
350
300
250
200
150
100
50
0
0.1
1
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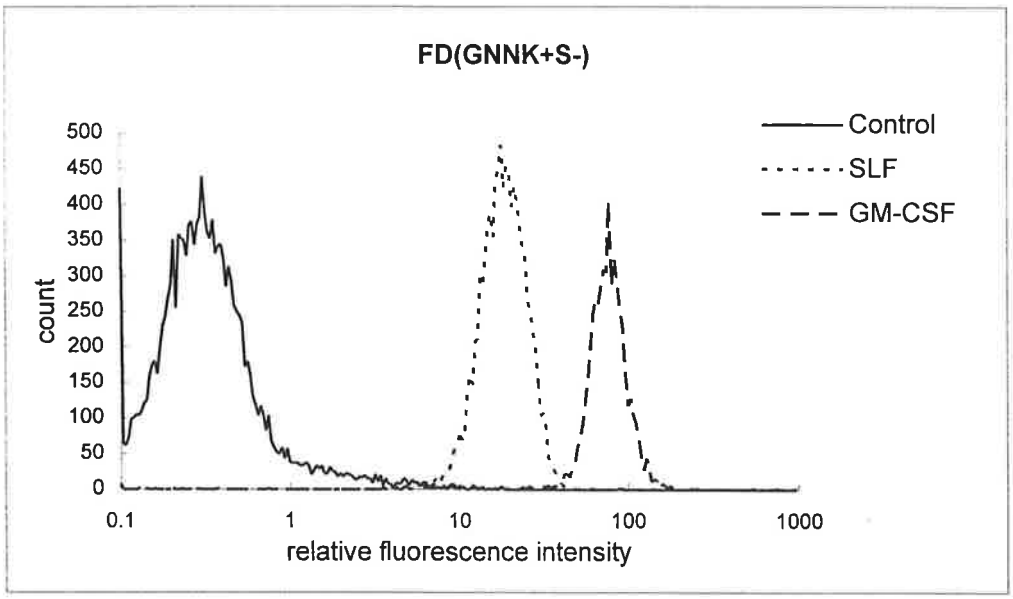
1000
relative fluorescence intensity


FD(GNNK+S-)
Control
SLF
GM-CSF
count
500
450
400
350
300
250
200
150
100
50
0
0.1
1
10
100
1000
relative fluorescence intensity

at ~ 1.8-fold (average) lower levels. At approximately 200 ng/ml M07e and FD(GNNK+S-) appeared to be approaching maximal proliferation in contrast to FD(GNNK-S+) and FD(GNNK+S+) cells. The control cells infected with the empty RufMC1*neo* retrovirus did not proliferate in hSLF demonstrating that hSLF was unable to stimulate the endogenous murine c-Kit receptor as has been previously reported by Lev *et al.*, (1993) (Figure 6.3.1).

Cells were also cultured in mSLF; the FDC-P1 infectants expressing human c-Kit proliferated at approximately the same rates in mSLF, except for the FD(GNNK+S+) cells which reproducibly demonstrated approximately 2-fold higher levels of proliferation in 100 ng/ml mSLF (Figure 6.3.2). The control cells showed 2.7, 2.5 and 6-fold lower levels of proliferation in mSLF in comparison to the FD(GNNK-S+), FD(GNNK+S-) and FD(GNNK+S+), respectively (Figure 6.3.2). Since the level of proliferation was higher in the FDC-P1 c-Kit infectants than the control FD(RufMC1*neo*) cells and the c-Kit infectants demonstrated dose-dependent proliferation in response to mSLF this suggested that mSLF was effective at stimulating the human and not just the murine c-Kit receptors (cross-species reactivity of murine SLF has been previously reported (Broxmeyer *et al.*, 1991; Lerner *et al.*, 1991). Generally the level of proliferation achieved by the cell lines in mSLF was slightly lower than that obtained in hSLF except for FD(GNNK+S+) cells which repeatedly produced 3-fold higher levels of proliferation in the presence of mSLF than in hSLF (Figure 6.3.2).

Although hSLF was shown to be inactive on the murine c-Kit receptor, the possibility of heterodimerization between the human and endogenous murine c-Kit receptors was not excluded in the above experiments. Therefore another murine factor-dependent cell line, 32D, which does not express endogenous c-Kit, was infected with the human c-Kit isoforms. 32D is an IL-3 dependent cell line, therefore infection was carried out in the same way as described for the FDC-P1 cells with murine IL-3 (mIL-3) (50 U/ml) being substituted for mGM-CSF. However unlike the FDC-P1 cells, only the 32D cells expressing the c-Kit(GNNK+S+) and c-Kit(GNNK-S+) isoforms could proliferate in hSLF alone. 32D(GNNK+S-) cells only proliferated in the presence of mIL-3 even though they expressed c-Kit. Table 6.3.2 contains the receptor numbers expressed by the 32D infectants expressing the various isoforms when cultured in mIL-3. 32D(GNNK-S-) cells again did not express c-Kit receptors.

Figure 6.3.1: Human SLF-dependent proliferation of the FD(GNNK+/-S+/-) infectants and M07e cells. FD(GNNK+/-S+/-) cells maintained in hSLF, FD(RufMC1*neo*) cells maintained in mGM-CSF and M07e cells maintained in hIL-3 were washed to remove residual cytokines. Cells were cultured in increasing concentrations of hSLF for 3 days in quadriplicate. Proliferation was determined by incorporation of ^{3}H-thymidine over the last 16 hours of culture. Data represented as the mean ± S.E.M.

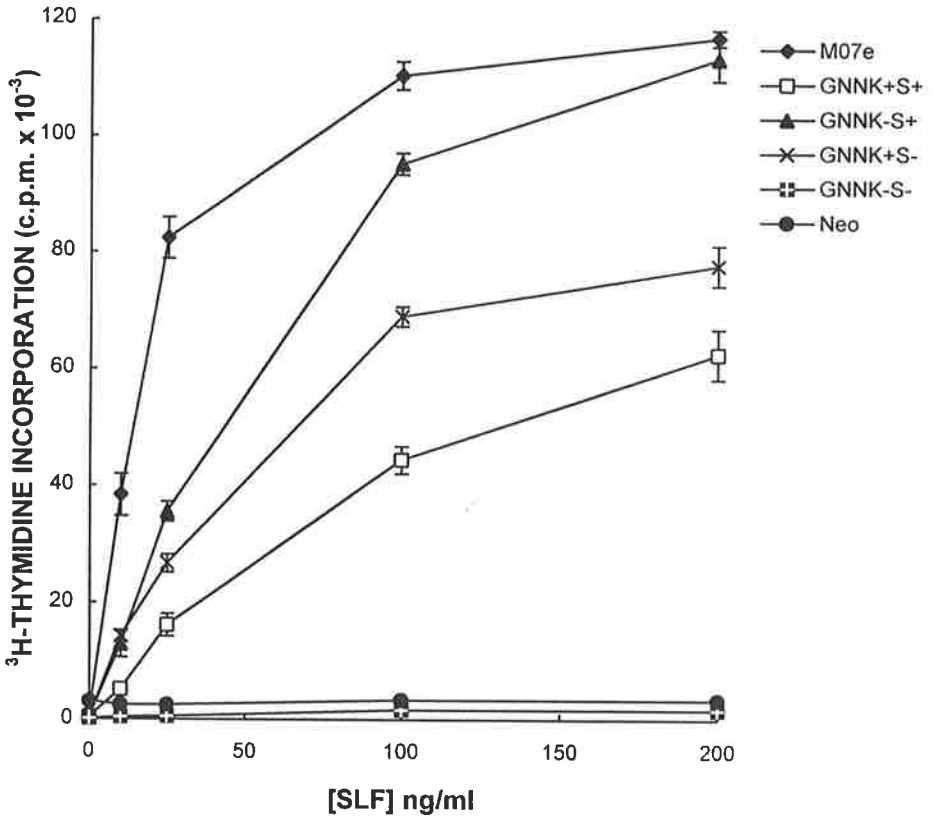
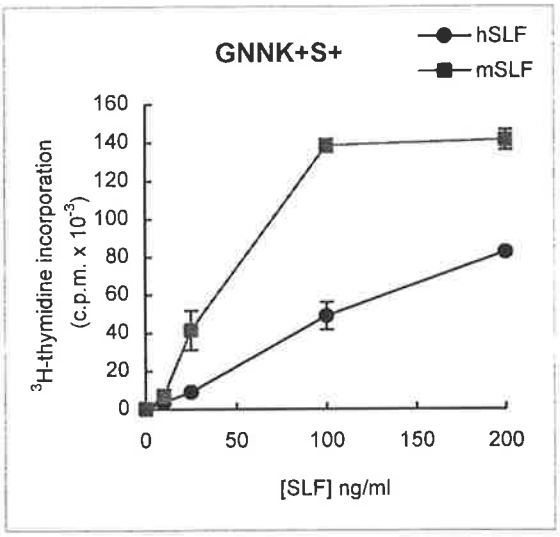
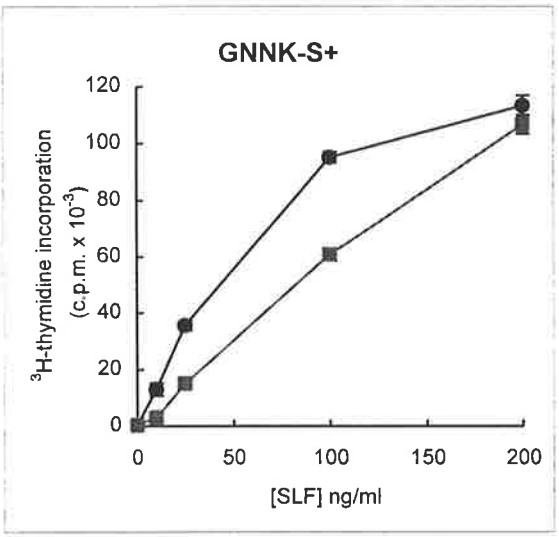
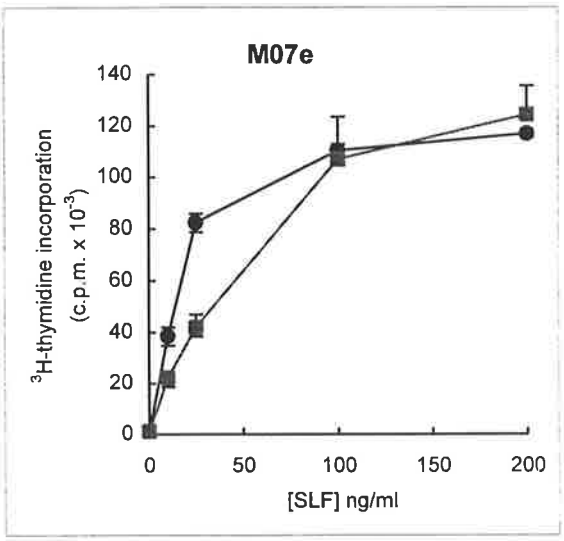
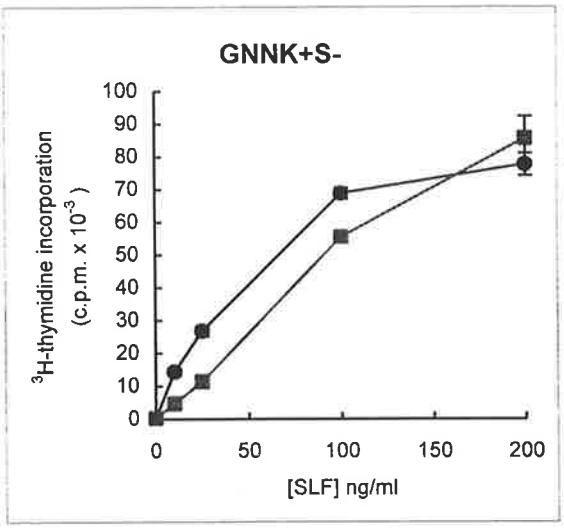

120
100
80
60
40
20
0
0
50
100
150
200
^{3}H-THYMIDINE INCORPORATION (c.p.m. x 10^{-3})
[SLF] ng/ml
M07e
GNNK+S+
GNNK-S+
GNNK+S-
GNNK-S-
Neo

Figure 6.3.2: Responsiveness of FDC-P1 cells expressing the c-Kit isoforms and M07e cells to human and murine SLF. FD(GNNK+S+/-) and FD(GNNK-S+) cells maintained in hSLF, FD(RUFMC1*neo*) cells maintained in mGM-CSF and M07e cells maintained in hIL-3 were washed and cultured in the presence of increasing concentrations of hSLF or mSLF for 3 days. Proliferation was determined by ^{3}H-thymidine incorporation in the last 16 hours of culture. Data represented as the mean ± S.E.M. of quadruplicate wells.


GNNK+S+
hSLF
mSLF
^{3}H-thymidine incorporation (c.p.m. x 10^{-3})
[SLF] ng/ml


GNNK-S+
^{3}H-thymidine incorporation (c.p.m. x 10^{-3})
[SLF] ng/ml


M07e
^{3}H-thymidine incorporation (c.p.m. x 10^{-3})
[SLF] ng/ml


GNNK+S-
^{3}H-thymidine incorporation (c.p.m. x 10^{-3})
[SLF] ng/ml

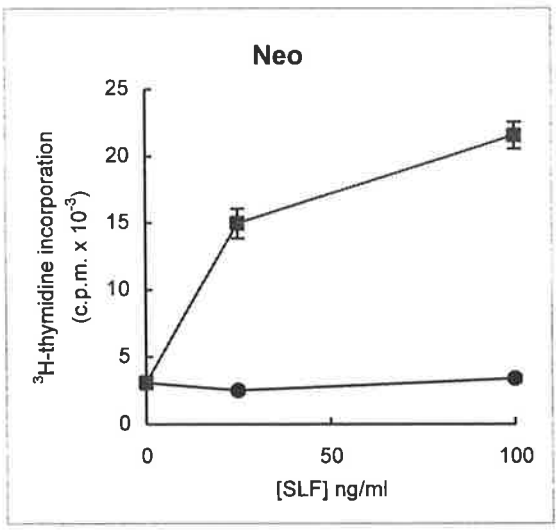

Neo
^{3}H-thymidine incorporation (c.p.m. x 10^{-3})
[SLF] ng/ml

Table 6.3.2: Receptor numbers expressed by the 32D(GNNK+/-S+/-) infectants cultured in mIL-3

FDC-P1 cell lines	*receptors/cell (x 10^3)
GNNK+S+	118
GNNK-S+	189
GNNK+S-	11.5
GNNK-S-	<1
RUFMC1*neo*	<1

* Cells maintained in hSLF were washed and cultured in 50 U/ml mIL-3 overnight. Receptor numbers were quantitated using QIFIKIT® beads (refer to section 2.2.4 for method).

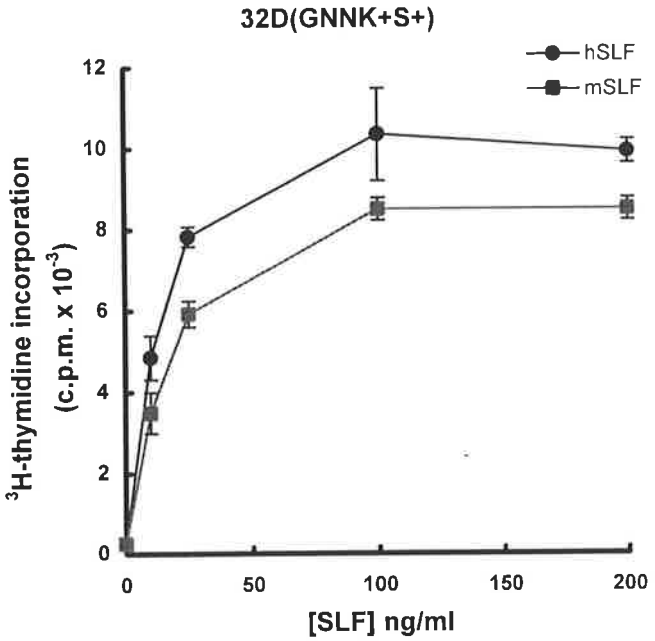
32D(GNNK+S-) cells expressed 10-16-fold lower receptor numbers than 32D(GNNK+S+) and 32D(GNNK-S+), respectively, which may explain why they did not proliferate in hSLF alone.

Proliferation of 32D(GNNK+S+) and 32D(GNNK-S+) cells in murine and human SLF demonstrated similar rates of ^{3}H-thymidine uptake in both factors (Figure 6.3.3). This was in contrast to the FD(GNNK+S+) cells which had demonstrated a 3-fold greater uptake in mSLF than in hSLF at 100 ng/ml as described above (Figure 6.3.2). It was possible therefore that the presence of the endogenous murine c-Kit receptor was contributing to this phenomenon. The level of ^{3}H-thymidine uptake by the 32D(GNNK+/-S+) infectants in 100 ng/ml hSLF compared to their FDC-P1 counterparts demonstrated that the former had a 5-10 fold lower uptake level even though they expressed 1.8-3 fold higher levels of c-Kit, respectively (comparison of results displayed in Figure 6.3.2 and 6.3.3; assays performed on the same day). One possibility for the lower proliferative capacity of the 32D cell lines may be due to the fact that these cells had mycoplasma which interferes with the ^{3}H-thymidine incorporation assay.

6.4 *The effect of anti-c-Kit mAb on the binding of hSLF and vice versa*

Cells were preincubated with either α-c-Kit mAb or the isotype-matched negative control mAb prior to incubation with biotin-labelled (bio)-hSLF which was detected by IIF and flow cytometry. The assay was performed at 4°C in the presence of sodium azide (NaN_3) in order to prevent receptor endocytosis. Bio-hSLF binding was not affected by preincubation with YB5.B8 on any of the cells analysed (Table 6.4.1). 1DC3 slightly inhibited the binding of bio-hSLF to all cell lines and reproducibly inhibited more strongly in the case of FD(GNNK+S-) cells. 17F11 enhanced the binding of bio-hSLF to M07e cells but showed no effect on the binding of bio-hSLF to any of the FDC-P1 cells expressing the various c-Kit isoforms. SR-1 had a dramatic effect almost completely abolishing the binding of bio-hSLF on all cells. The reciprocal experiment, demonstrated that preincubated with 200 ng/ml hSLF mostly influenced the binding of YB5.B8 and SR-1. hSLF slightly reduced the binding of

Figure 6.3.3: Responsiveness of 32D cells expressing the c-Kit(GNNK+/-S+) isoforms to human and murine SLF. 32D(GNNK-/+S+) cells maintained in hSLF were washed and cultured in the presence of increasing concentrations of hSLF or mSLF for 3 days. Proliferation was determined by ^{3}H-thymidine incorporation in the last 16 hours of culture. Data are represented as the mean cpm ± S.E.M. of triplicate wells.


32D(GNNK+S+)
hSLF
mSLF
^{3}H-thymidine incorporation
(c.p.m. x 10^{-3})
0
2
4
6
8
10
12
0
50
100
150
200
[SLF] ng/ml

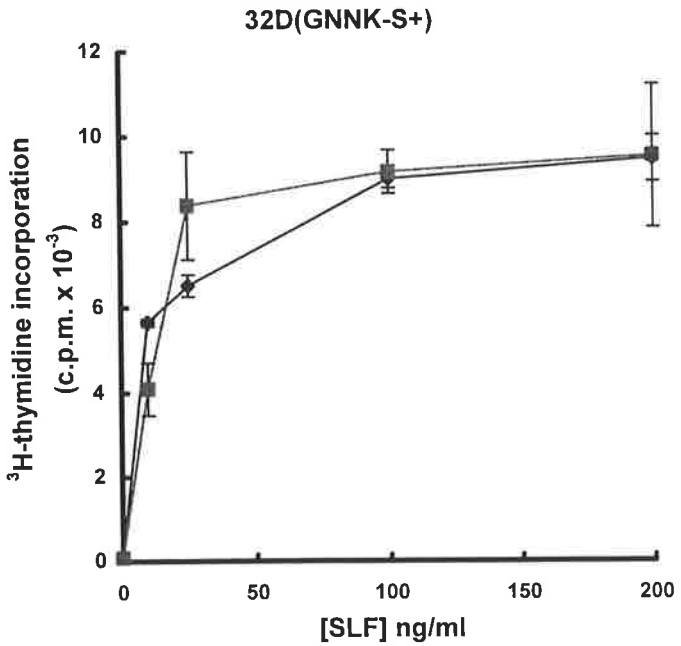

32D(GNNK-S+)
^{3}H-thymidine incorporation
(c.p.m. x 10^{-3})
0
2
4
6
8
10
12
0
50
100
150
200
[SLF] ng/ml

Figure 6.4.1: The effect of anti-c-Kit mAb on the binding of biotin-labelled hSLF to the FD(GNNK+/-S+/-) and M07e cells.

	Relative mean fluorescence intensity			
MAb	M07e	FD(GNNK+S+)	FD(GNNK+S-)	FD(GNNK-S+)
Control	100	100	100	100
YB5.B8	91	94	106	101
1DC3	67	67	30	58
17F11	130	98	84	101
SR-1	4	11	7	12

Cells were washed and cultured overnight in medium containing mGM-CSF prior to incubation with α-c-Kit mAb (20 μg/ml final of purified YB5.B8 or 1DC3 ; 1/5 final dilution of 17F11 hybridoma culture supernatant; 1/20,000 final of SR-1 ascites) or isotype-matched negative control mAb for 30 minutes on ice in the presence of 0.01% NaN_3 followed by the addition of biotin-labelled hSLF. After a further 30 minutes on ice, cells were stained with strepavidin-PE and analysed by flow cytometry. The results are represented as the mean fluorescence intensity relative to that observed with cells preincubated with the isotype-matched negative control mAb, assigned the arbitary value 100. Data represents typical results obtained from one of three experiments.

Table 6.4.2: The effect of hSLF on the binding of anti-c-Kit mAb to the FD(GNNK+/-S+/-) and M07e cells.

mAb	Relative mean fluorescence intensity			
	M07e	FD(GNNK+S+)	FD(GNNK+S-)	FD(GNNK-S+)
No SLF	100	100	100	100
YB5.B8	43	47	47	33
1DC3	90	81	72	75
17F11	144	166	117	179
SR-1	19	39	45	29

Cells were washed and incubated in medium containing mGM-CSF overnight prior to incubation in medium or with hSLF (200 ng/ml) for 10 minutes on ice in the presence of 0.01% NaN_3. Cells were then incubated with the indicated mAb (5 μg/ml final of purified YB5.B8 or IDC3; 1/5 final dilution of 17F11 hybridoma culture supernatant; 1/20,000 final of SR-1 ascites) or isotype-matched control antibody for a further 30 minutes on ice. The cells were then stained with FITC-conjugated α-mouse Ig and analysed by flow cytometry. For each antibody the mean fluorescence intensity observed with cells preincubated with hSLF is expressed relative to the control (no SLF), assigned the arbitary value 100. Data represents typical results obtained from one of three experiments.

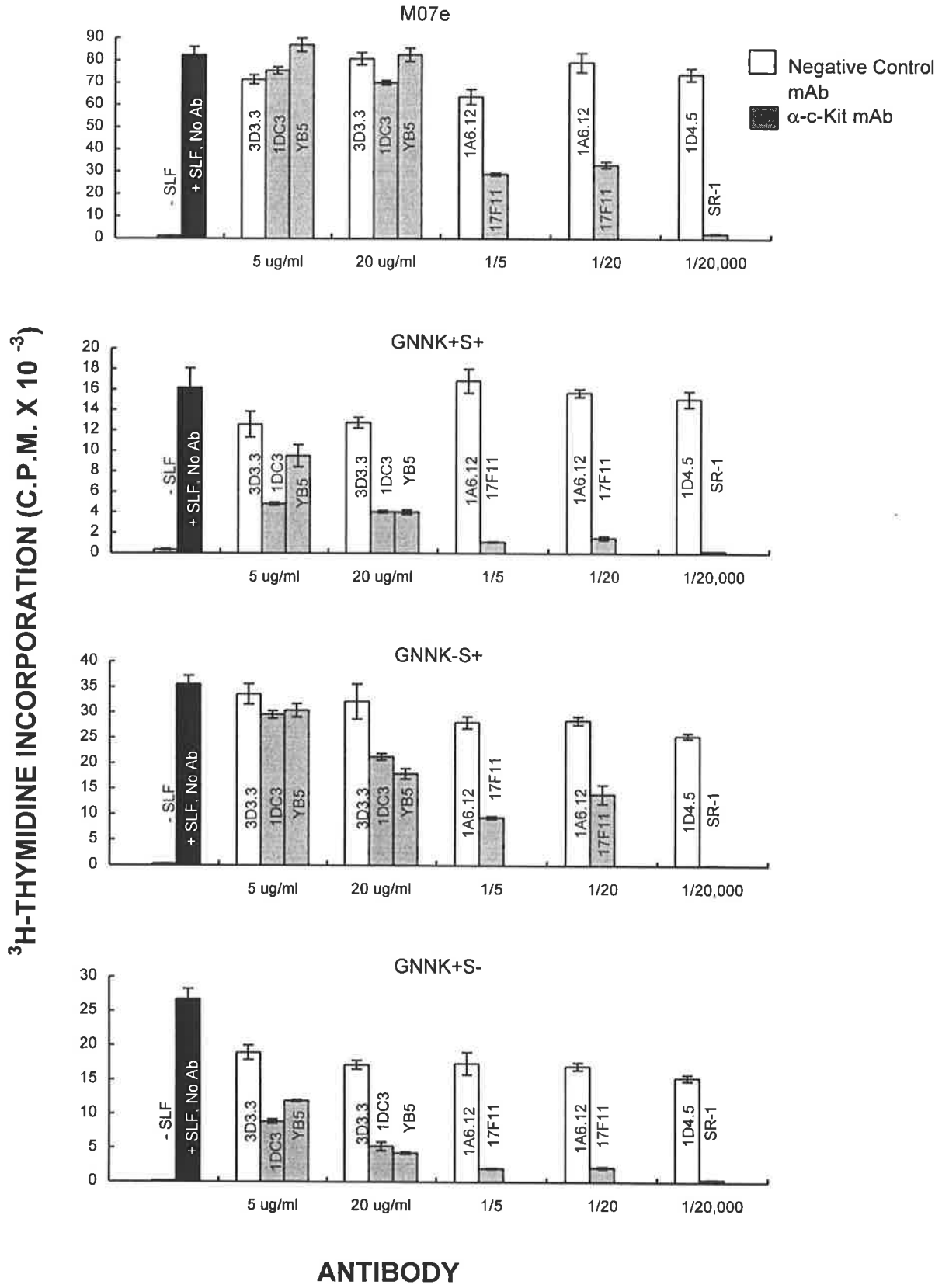
1DC3 and enhanced the binding of 17F11 (Table 6.4.2). Results presented in Tables 6.4.1 and 6.4.2 were reconfirmed in repeat experiments.

6.5 *The ability of anti-c-Kit antibodies to block SLF-dependent proliferation of FDC-P1 cells expressing the human c-Kit isoforms.*

FDC-P1 cells expressing the human c-Kit isoforms which had been maintained in hSLF were washed and assayed in medium containing 25 ng/ml hSLF and the α-c-Kit mAb indicated or the isotype-matched negative control mAb. Proliferation was assessed by uptake of ^{3}H-thymidine after 3 days of culture. M07e cells previously maintained in human IL-3 (hIL-3) were also included in the assay as a positive control. Proliferation of M07e cells was completely inhibited by SR-1 and by approximately 50% by 17F11, in comparison to the proliferation achieved in cultures containing the equivalent isotype-matched negative control mAb. 1DC3 did not inhibit proliferation at 5 μg/ml but gave a slight inhibition of approximately 15% at 20 μg/ml. YB5.B8 as expected did not inhibit the proliferation of M07e cells at either concentration (Figure 6.5.1).

Interestingly, the antibodies affected the proliferation of the FDC-P1 infectants in an isoform specific manner. All the FDC-P1 infectants were completely inhibited by SR-1. Both FD(GNNK+S+) and FD(GNNK+S-) cells were inhibited by approximately 91.5% and 88% by 17F11, respectively, while FD(GNNK-S+) proliferation was inhibited by 51%. The proliferation of both FD(GNNK+S+) and FD(GNNK+S-) cells was inhibited by an average of 68% and 72%, respectively, by 1DC3 and YB5.B8 at 20 μg/ml. Although 1DC3 and YB5.B8 were able to inhibit to a similar extent at 20 μg/ml, at the lower concentration of 5 μg/ml 1DC3 was reproducibly more effective than YB5.B8. The most striking differences seen between the FDC-P1 cells expressing the c-Kit isoforms was the lack of significant inhibition by mAb 1DC3 and YB5.B8, at 5 μg/ml, on the hSLF-driven proliferation of FD(GNNK-S+) cells. This was equivalent to the result displayed by these mAb, used at this concentration, on M07e cells. However, in contrast to the M07e cells 1DC3 and YB5.B8 at 20 μg/ml were able to inhibit FD(GNNK-S+) proliferation by approximately 34 and 44%, respectively (Figure 6.5.1). Similar experiments were performed on the FD(GNNK-S-) cells, however

Figure 6.5.1: Effect of a panel of anti-c-Kit mAb on hSLF-driven proliferation of FDC-P1 cells expressing the c-Kit isoforms compared to M07e cells. Cells were washed to remove residual cytokines used to maintain them in and cultured in 25 ng/ml hSLF in the presence of the α-c-Kit mAb: purified YB5.B8 (IgG_1) (at 5 and 20 μg/ml final), 1DC3 (IgG_1) (at 5 and 20ug/ml final), 17F11 (IgM) (hybridoma supernatant at 1/5 and 1/20 final) and SR-1(IgG_{2a}) (ascites at 1/20,000 final) or the isotype-matched negative control mAb at the equivalent concentrations: 3D3.3 (IgG_1), 1A6.12 (IgM) and 1D4.5 (IgG_{2a}). Proliferation was monitored by ^{3}H-thymidine incorporation in the last 16 hours of the 3 day assay. Data represents typical results obtained from one of three experiments portrayed as the mean ± S.E.M. of quadruplicate wells.


M07e
GNNK+S+
GNNK-S+
GNNK+S-
Negative Control mAb
α-c-Kit mAb
- SLF
+ SLF, No Ab
3D3.3
1DC3
YB5
1A6.12
17F11
1D4.5
SR-1
5 ug/ml
20 ug/ml
1/5
1/20
1/20,000
3H-THYMIDINE INCORPORATION (C.P.M. X 10 -3)
ANTIBODY

none of the mAb were able to inhibit proliferation, not even the potent mAb SR-1, further demonstrating that these cells were growing due to an event unrelated to c-Kit expression (data not shown).

6.6 DISCUSSION

The FDC-P1 cells independently expressing the c-Kit variants differed in their responsiveness to hSLF. FD(GNNK-S+) cells had an increased proliferative capacity (2-fold) relative to FD(GNNK+S+) cells expressing comparable levels of receptors. NIH/3T3 cells independently expressing the murine c-Kit(GNNK-/+) isoforms have similarly shown that c-Kit(GNNK-) expressing cells are more responsive to the mitogenic signal induced by mSLF and that this is associated with enhanced tyrosine kinase activity compared to those cells expressing the c-Kit(GNNK+) receptor (Blouin and Bernstein, unpublished; referred to in Piao *et al.*, 1994; Reith *et al.*, 1991). The hSLF-dependent proliferation results obtained in this chapter also suggest the possibility that the c-Kit isoforms differ in their affinity for hSLF (Figure 6.3.1). The enhanced mitogenic response achieved by the c-Kit(GNNK-S+) isoform may therefore be due to an increased affinity for hSLF and/or enhanced tyrosine kinase activation resulting in a more rapid turnover of the c-Kit(GNNK-S+) protein. The latter may explain why c-Kit(GNNK-S+) displayed a 2-fold greater downregulation of surface protein compared to c-Kit(GNNK+S+/-) when cells were cultured in hSLF rather than mGM-CSF (Figure 6.2.3). Binding of the detecting α-c-Kit mAb 1DC3 to both c-Kit(GNNK+/-) isoforms was minimally affected by preincubation of the cells with hSLF (200 ng/ml) (Table 6.4.2); thus blocking of 1DC3 binding by hSLF does not account for these results. The reduced signal seen on FD(GNNK-) cells cultured in hSLF was therefore most likely due to a more rapid rate of SLF-mediated internalization of the receptor. Analysis of the relative affinities of the isoforms for hSLF (using Scatchard analysis), their kinetics of tyrosine kinase activation and receptor internalization are the subject of future studies to be carried out in the laboratory which will expand on the results obtained in this chapter. FD(GNNK+S-) cells also displayed a higher rate of proliferation compared to FD(GNNK+S+) cells however this could

be due to the higher receptor concentration expressed by the former and was not necessarily due to the deletion of the serine residue.

It was also observed that hSLF-dependent proliferation of the FD(GNNK-S+) cells could not be inhibited as effectively as FD(GNNK+S+/-) cells by the α-c-Kit mAb YB5.B8, 1DC3 and 17F11. This implied that the deletion/insertion of GNNK was contributing to the differences described previously (section 6.1). Interestingly, the degree of inhibition of hSLF-driven proliferation of M07e cells by 17F11, SR-1, YB5.B8 (5 μg/ml) and 1DC3 (5 μg/ml) were almost identical to that achieved on FD(GNNK-S+) proliferation. Since M07e cells co-express the various isoforms with predominance of the c-Kit(GNNK-) and c-Kit(S+) containing isoforms (Crosier *et al.*, 1993) it is possible that in M07e cells hSLF-driven proliferation is governed by c-Kit(GNNK-S+). At higher concentrations of YB5.B8 and 1DC3 (20 μg/ml) partial inhibition of SLF-dependent FD(GNNK-S+) proliferation was seen, in contrast to M07e cells. This may be due to the extreme difference in receptor levels expressed by FD(GNNK-S+) (~6.5 x 10^4 receptors/cell) compared to M07e cells (~3.6 x 10^5 receptors/cell; Cole *et al.*, 1996) and/or due to the possibility that heterodimerization could occur between the different c-Kit isoforms co-expressed by these cells.

Inhibition of hSLF-dependent proliferation of the cells by the antibodies can occur via a number of mechanisms. The mAb may inhibit proliferation by competing for the ligand binding site resulting in complete or partial loss of SLF binding; by inducing a conformational change affecting ligand binding; or by preventing subsequent ligand mediated events involved in transmitting a mitogenic signal, such as receptor dimerization. Complete inhibition of hSLF-driven proliferation by SR-1 correlated with the ability of this antibody to almost completely block the binding of bio-hSLF to all the cell lines. Preincubation of the cells with hSLF also interfered with the binding of SR-1, and together these results suggest that SR-1 is competing with hSLF for binding to the receptor and as a result is able to inhibit proliferation. This conforms with the antagonistic ability of this mAb described by Broudy *et al.*, (1992) and Ashman *et al.*, (1994).

The partial inhibition of proliferation achieved by 17F11 was not due to interference in the binding of hSLF as preincubation with 17F11 did not generally affect the binding of

bio-hSLF, nor did hSLF inhibit the binding of 17F11. In fact the latter enhanced the binding of 17F11 to the cells possibly due to an increase in the affinity of the antibody as a result of SLF-mediated receptor oligomerization. These results suggest that 17F11 may be affecting subsequent SLF-mediated events necessary for transmission of a signal through the receptor such as dimerization. Preliminary studies have demonstrated that in the murine system the c-Kit(GNNK-) isoform had an increased capacity to dimerize in the presence of mSLF compared to the GNNK+ isoform (Blouin and Bernstein, unpublished). In this study the less effective inhibition of 17F11 on FD(GNNK-S+) and M07e SLF-driven proliferation (~50%) compared to FD(GNNK+S+/-) (~90%) may be influenced by the differential dimerization capabilities of these isoforms, possibly as a result of differential conformational changes. Comparative analysis of the dimerization capabilities of the human c-Kit isoforms expressed in the FDC-P1 cell background will be addressed in future studies. 17F11 has been shown to display slight agonist activity (Ashman *et al.*, 1994) therefore partial proliferation of the cell lines in the presence of 17F11 may also be attributed to this, possibly as a result of partial mimicry of hSLF binding to the receptor.

The mAb, 1DC3, had a slight inhibitory effect on the binding of bio-hSLF to all cells analysed and the reciprocal experiment showed that 1DC3 binding was not greatly influenced by preincubation with hSLF. These results suggest that hSLF-driven proliferation of the cells was inhibited due to other events required to transmit a signal rather than ligand binding. The slight inhibition of bio-hSLF binding to the receptor by 1DC3 demonstrates that the epitope of 1DC3 is in close proximity to the ligand binding domain or that a SLF-induced conformational change affects 1DC3 binding. 1DC3 binds to the first Ig-like domain of the c-Kit receptor (D. Givol & S. Lev, through personal communication). This domain has been demonstrated to be involved in stabilising the binding of hSLF to the ligand binding site located in domain 2 (Lev *et al.*, 1993), therefore 1DC3 may affect this stabilisation.

Preincubation of cells with YB5.B8 generally had no effect on the binding of bio-hSLF on any of the cells analysed. However preincubation with hSLF was able to significantly interfere with the binding of YB5.B8 to the cells. As with the 1DC3 mAb, these results similarly suggest that YB5.B8 inhibition of hSLF-driven proliferation is not due to the

lack of hSLF binding. YB5.B8 has been mapped to domain 2 (D. Givol & S. Lev, through personal communication) which is also the location of the ligand binding site. The close proximity of binding of these two molecules may therefore explain the interference of YB5.B8 binding by hSLF. It is notable however that although YB5.B8 and SR-1 binding were effected by preincubation with hSLF these mAb bind to different c-Kit epitopes as they do not crossblock one another (Broudy *et al.*, 1992; Ashman *et al.*, 1994). The lack of or slight inhibition of M07e and FD(GNNK-S+) cells, respectively, by YB5.B8 and 1DC3 compared to the more efficient inhibition achieved on FD(GNNK+) cells does not appear to be due to obvious changes in their antibody or ligand binding characteristics.

Based on the data presented in this chapter the discrepancy between the inhibitory action of YB5.B8 in several SLF-dependent culture systems of normal haemopoietic cells described in section 6.1 (Cambareri *et al.*, 1988; Ashman *et al* 1990; Cicuttini *et al.*, 1992; A. Zannettino., unpublished data; Matos *et al.*, 1993) compared to the lack of inhibition of hSLF-dependent proliferation of M07e cells (Ashman *et al.*, 1994; and Figure 6.5.1) could partially be explained if the normal haemopoietic cells in these systems predominantly expressed the c-Kit(GNNK+) isoform. Although predominance of the c-Kit(GNNK-) isoform has been reported at the RNA level in unfractionated normal bone marrow cells (Crosier *et al.*, 1993 and Piao *et al.*, 1994) it is possible that the c-Kit isoforms are regulated during the differentiation of haemopoietic cells (as suggested by Piao *et al.*, 1994). If this were the case cells at various stages of differentiation might predominantly express the c-Kit(GNNK+) variant and as a consequence might result in a different biological outcome compared to cells predominantly expressing c-Kit(GNNK-). Other factors which may contribute to the differences in YB5.B8 inhibition in the systems described above may include the degree of heterodimerization that may occur between co-expressed isoforms; differences in c-Kit glycosylation in different cell types; or whether SLF-driven proliferation was induced by soluble versus membrane-bound SLF.

Murine SLF has the ability to cross-react with the human c-Kit receptor (Lerner *et al.*, 1991; Broxmeyer *et al.*, 1991). The control FD(RUFMC1*neo*) cells expressing only endogenous murine c-Kit displayed a lower proliferative capacity in mSLF than any of the

FDC-P1 human c-Kit expressing cells, suggesting that in mSLF the latter were at least in part using the human c-Kit receptor. Control cells did not respond to hSLF demonstrating the specificity of this ligand (Lev *et al.*, 1993). mSLF generally induced slightly lower levels of proliferation than hSLF, (except for FD(GNNK+S+) cells), which is not surprising due to the lower affinity of the human c-Kit receptor for mSLF compared to hSLF. The reproducible enhanced (3-fold) rate in FD(GNNK+S+) cell proliferation in mSLF (100 ng/ml) may have been due to the expression of murine c-Kit, since 32D cells which lacked endogenous c-Kit did not display this enhanced proliferation. This may be explained by the data demonstrated by Lev *et al.*, (1992 b) where the affinity of rat SLF binding to the human c-Kit receptor was increased in cells co-expressing murine and human c-Kit receptors. Why this was only demonstrated by the c-Kit(GNNK+S+) isoform in this chapter is presently unclear.

It is possible that in the FDC-P1 cells, co-dimerization of the ectopic human and endogenous murine c-Kit receptors may occur in the presence of mSLF due to its ability to bind to both the human and murine receptors. Since the various isoforms have been found to be co-expressed in tissues and cells it is most likely that the FDC-P1 cells also co-express both the murine c-Kit(GNNK+/-S-) isoforms. It is therefore possible that co-dimerization of either murine isoform with the human c-Kit(GNNK+S+) is occurring and that this could account for the increase in proliferation seen by FD(GNNK+S+) cells in mSLF. It is noteworthy that this effect was not seen in the 32D(GNNK+S+) cells which don't express endogenous murine c-Kit. It was also observed that FD(GNNK-S+) cells were able to proliferate at an approximate 2.3-fold higher rate than FD(GNNK+S+) cells, however this was not seen in the 32D(GNNK-S+) cells; therefore the presence of murine c-Kit in the FD(GNNK-S+) may also be contributing to its proliferative capacity in hSLF. According to the dimerization model put forward by Yarden and colleagues (Lev *et al.*, 1992 b; Blechman *et al.*, 1995) which does not depend on the bivalency of the SLF molecule to bring about oligomerization, it is possible for murine and human c-Kit receptor subunits to dimerize in the presence of the species specific hSLF. The ability of the human c-Kit isoforms to co-dimerize with endogenous murine c-Kit in the presence of murine or human SLF are currently being investigated in our laboratory.

CHAPTER 7

General Discussion

7. GENERAL DISCUSSION

The association of the genes encoding c-Kit and its ligand, SLF, with the mutations underlying the phenotypes of the *W* and *Sl* mutant mice, respectively, demonstrated the importance of this receptor/ligand complex during haemopoiesis. Of particular importance to the work described in Chapter 3, was the discovery that SLF was produced as both a membrane-associated and soluble factor. Sl/Sl^d mice demonstrated that these isoforms of SLF differ qualitatively and/or quantitatively in their ability to support haemopoietic stem cells and mast cells, since the production of only soluble SLF by these mice could not compensate for the loss of membrane-associated SLF. Similarly, *in vitro* co-culture of c-Kit expressing cells such as mast cells, stem cells, megakaryocytes (Fujita *et al.*, 1989; Adachi *et al.*, 1992; Toksoz *et al.*, 1992; Avraham *et al.*, 1992) and as described in Chapter 3, FDC-P1 cells, with feeder layers reflected the phenotype seen *in vivo*. Feeder layers expressing membrane-bound SLF had the ability to support cells in a contact-dependent and GM-CSF-independent manner, in contrast to those secreting only the soluble form of SLF which failed to support cell survival and/or proliferation.

The differential response of FDC-P1 cells to membrane-bound versus soluble SLF (Caruana *et al.*, 1993) may be due to the quantitative difference between the two forms of SLF ie. the levels of soluble SLF produced by the feeder layers are low and diffusible in comparison to the high localised concentration provided by membrane-bound SLF. However, it has recently been reported that membrane-bound SLF is able to cause a more prolonged activation of the c-Kit receptor in contrast to soluble SLF. This occurs due to the inability of c-Kit/membrane-bound SLF complex to be internalised and degraded therefore prolonging its half life, in contrast to soluble SLF which induces c-Kit internalisation and acts as a c-Kit downregulator (Miyazawa *et al.*, 1995). It is possible therefore that high levels of soluble SLF in certain systems (Jozaki *et al.*, 1991; Zsebo *et al.*, 1990a) may be able to provide a similar continued stimulation to that produced by membrane-bound SLF. However, the fact that soluble SLF can downregulate c-Kit (Miyazawa *et al.*, 1995) portrays the qualitative difference between these two forms.

High concentrations of soluble recombinant mSLF alone were unable to compensate for membrane-bound SLF in supporting the proliferation of FDC-P1 cells in culture. The addition of low levels of mGM-CSF, however, allowed for the selection of FDC-P1(SLF) cells which were capable of proliferating in mSLF alone, possibly as a result of the potent synergism which was demonstrated to occur between these factors at sub-optimal levels of mGM-CSF. Synergism as well as the upregulation of c-*kit* mRNA and c-Kit receptors in low levels/or in the absence of mGM-CSF (Caruana *et al.*, 1993; Welham and Schrader 1991), may have determined the responsiveness of the cells to high levels of soluble mSLF. An increase in receptor density would result in the availability of more surface receptors for stimulation by mSLF, therefore amplifying subsequent intracellular signalling leading to a proliferative response of FDC-P1(SLF) cells. It has recently been reported that the 'strength of signal' generated by the EGF or insulin receptors, as a consequence of the variation of receptor density, can control the nature of the biological response of the cell. Stimulation of endogenous levels of the EGF/insulin receptors results in transient activation of mitogen-activated protein kinase (MAPK) resulting in the proliferation of the cells. Overexpression of these receptors results in sustained ligand-mediated activation of MAPK and its translocation to the nucleus which corresponds to the differentiation of the cells (Traverse *et al.*, 1994; Dikic *et al.*, 1994; reviewed by Marshall *et al.*, 1995). The 'strength of signal' concept could therefore explain the differential responsiveness of FDC-P1(SLF) versus FDC-P1(GM) or FDC-P1(IL-3) cells to soluble mSLF due to their differences in receptor density. Similarly it can explain the differential responsiveness of cells to membrane-bound versus soluble SLF described in this study and others (Fujita *et al.*, 1989; Toksoz *et al.*, 1992) which also corresponds to sustained versus transient c-Kit tyrosine kinase activation (Miyazawa *et al* 1995).

The results described in Chapters 4 and 5, demonstrated that receptor levels also play an important role in the transforming capability of the unaltered c-*kit* proto-oncogene product. Analysis of clones expressing increasing levels of murine c-*kit* mRNA and c-Kit surface protein, described in Chapter 4, demonstrated a significant correlation ($R=0.53$, $p<0.01$) between c-*kit* mRNA expression and factor-dependent, anchorage independent growth. Low

expressing clones, NIH(mu*kit*).R31-R49, generally were unable to grow in an anchorage independent manner. Clones expressing intermediate levels of c-*kit*, NIH(mu*kit*).R33-R65, resulted in transformation and this generally increased in its extent as the level of c-*kit* increased, as displayed by the NIH(mu*kit*).R55-R56 group of clones. Other characteristics indicative of transformation were similarly influenced by increased c-*kit* expression levels. For example epNIH(mu*kit*)-R4 cells, expressing 2.3-fold higher c-*kit* mRNA levels than epNIH(mu*kit*)-R2 cells, displayed an enhanced transformed morphology as well as the ability to induce more rapid tumours in *nude* mice. Increased receptor expression of the human c-Kit isoforms, under the control of the RUFMC1Neo LTR described in Chapter 5, resulted in an increase in the production of factor-dependent foci. In contrast to this however, anchorage independent colony formation appeared to be inhibited as the density of human c-Kit receptors increased. This was also demonstrated with the c-Kit(GNNK+S+) isoform when progressively amplified due to resistance of cells to increasing concentrations of Mtx. Unselected NIH(C3A3) cells expressing the lowest level of c-Kit(GNNK+S+) were unable to produce colonies, however a 2-fold increase in c-Kit expression by NIH(C3A3)$_{200\ nM}$ cells resulted in optimal transformation, with further amplification resulting in partial or complete inhibition of anchorage independent growth. In light of these data, it is possible that similar mechanisms resulting in inhibition of anchorage independent growth applied to the clones NIH(mu*kit*).R62 and R56, described in Chapter 4, which produced low or negligible numbers of colonies in soft agar despite their high level of murine c-*kit* mRNA and c-Kit protein. The anchorage independent growth displayed by the NIH(C3A3) series of cell lines together with those of the NIH(mu*kit*).R31-R56 clones revealed that a 'window' of c-Kit expression existed that was necessary for maximal induction of anchorage independence.

Although not analysed in this study, it is most likely that induction of anchorage independent growth by cells expressing intermediate levels of c-Kit, in contrast to low levels, is a consequence of the amplification of signalling generated by the increase in c-Kit receptor expression. Therefore the 'strength of signal' theory described above may also explain the increase in transforming potential of the c-Kit receptor as its level of expression increases. High levels of c-Kit which resulted in the inhibition of anchorage independent growth

(for example in the human c-Kit systems and NIH(mu*kit*).R56 and R62) may be a consequence of over activation of the receptor. Under these circumstances a disruption in the balance and/or specificity of signalling molecules may inhibit/turn off the events required to stimulate anchorage independent growth. It has been reported that cells expressing high levels of the EGFR results in inhibition of EGF-driven proliferation and that this was associated with an increase in tyrosine kinase activity of the receptor (Kawamoto *et al.*, 1984; Riedel *et al.*, 1987). Future analysis of the level of tyrosine kinase activation of the human c-Kit receptor and subsequent phosphorylation/association of downstream signalling molecules, in NIH/3T3 cells expressing different levels of c-Kit, may provide an explanation for the transformation results obtained in this study. Alternatively, reduced anchorage independent growth may be related to the reduced levels of c-*kit* mRNA expressed in these cells despite their increased level of c-Kit surface protein, although this was only demonstrated in the human c-Kit expressing cells. The reason for the contrasting results demonstrated by the focus-formation and anchorage independence assays with cells expressing the human c-Kit isoforms is not clear at present, but may suggest that different threshold levels of c-Kit expression are required to induce the various phenotypes characteristic of transformation. In light of recent data, demonstrating dissociation between anchorage independence and focus-formation induced by the CSF-1\αPDGF receptor hybrid (Yu *et al.*, 1995), it is possible that c-Kit receptor densities influence downstream signalling events that are differentially involved in producing these two transformation characteristics.

The levels of c-Kit receptor expression obtained in this study for the human and murine receptors, demonstrated that murine c-Kit was expressed at higher than physiological levels in the NIH(mu*kit*) pool, as determined at the mRNA level (discussed in more detail in Chapter 4). NIH(mu*kit*) cells had approximately 20-fold higher c-Kit receptor levels than epNIH(GNNK+/-S+).2 cells, which expressed the highest levels of human c-Kit obtained in this study (~1.9×10^4 receptors/cell), comparable to physiological levels (described in more detail in Chapter 5). Based on transformation measured by anchorage independent growth, it was shown that in the human system that approximately 4×10^3 receptors/cell, as expressed by $NIH(C3A3)_{200\ nM}$ cells, were required prior to the induction of transformation by

c-Kit(GNNK+S+). However in the murine system, it appeared that clones expressing intermediate levels of c-Kit, NIH(mu*kit*).R33-R65, were required which expressed comparable levels to the NIH(mu*kit*) pool. Thus it was shown in this study that murine c-Kit receptor overexpression above physiological levels was required for transformation of NIH/3T3 cells but not essential for induction by the human c-Kit receptor. In comparison to the levels of expression required for the induction of transformation by other unaltered receptor tyrosine kinases (RTK), it has been shown that NIH/3T3 cells expressing up to 3.3×10^4 murine CSF-1R receptors/cell could induce transformation (Rohrschneider *et al.*, 1989) and up to 4×10^5 receptors/cell for the human EGFR (Velu *et al.*, 1987). The exact level of receptor expression essential for the induction of transformation was not addressed in these studies and it is quite possible that lower levels may have also induced transformation. Although Hudziak *et al.*, (1987), demonstrated that progressive amplification of the HER-2/*neu* receptor resulted in an increase in transformation, they did not quantitate the levels of receptor expressed, making it difficult to compare these levels with those obtained by the NIH(C3A3) series of cells, in which c-Kit was amplified using the analogous Mtx selection method.

The fact that expression of many of these unaltered RTK above a threshold level, results in transformation of indicator cell lines (Hudziak *et al.*, 1987, Di Fiore *et al.*, 1987; Velu *et al.*, 1987; Roussel *et al.*, 1987; Rohrschneider *et al.*, 1989; Alexander *et al.*, 1991; and Chapter 4 and 5) suggests that transformation may be the result of the over activation of a common signalling molecule(s). For example the c-*src* product which is a non-transforming tyrosine kinase is commonly activated by growth factor receptors. Elevated levels of c-*src* are commonly found in cells possessing high levels of the EGF or HER-2/*neu* receptors in tumours (Luttrell *et al.*, 1994; Muthuswamy *et al.*, 1994) and it has been shown that c-*src* can co-operate with the EGFR to induce more enhanced oncogenesis (Maa *et al.*, 1995). The tyrosine residue Y_{568} in the human c-Kit receptor has been shown to be conserved in the RTK type III subclass as a binding site for c-*src* family tyrosine kinases (reviewed in Herbst *et al.*, 1995a). Future analysis by our group of the phosphorylation/association of signalling molecules associated with the c-Kit receptor when expressed at different densities may reveal

similar co-operations. It should be noted here that ectopic expression of c-Kit in *Sl/Sl* cells did not induce transformation even in the addition of mSLF as had occurred in NIH/3T3 cells. NIH/3T3 cells are commonly used to assay the transforming potential of c-*oncs* and v-*oncs* due to their susceptibility to transformation. Thus, this may also be a result of the co-operation between the introduced gene and oncogenes already activated in NIH/3T3 cells during immortalisation (Land *et al.*, 1983a) which are not found in *Sl/Sl* cells.

Anchorage independent growth and/or focus-formation was obtained by all pool populations of NIH/3T3 cells expressing either murine or human c-Kit in the absence of exogenously added SLF. For both species of c-Kit receptors this may be attributed to intracellular autocrine stimulation of c-Kit by endogenously produced SLF (discussed in more detail in Chapter 4). An increase in transformation in the absence of added mSLF was generally obtained as the level of c-Kit increased (eg NIH(mu*kit*)-R2 versus NIH(mu*kit*)-R4 Chapter 4 or focus-formation of NIH(GNNK+/-S+/-) cells, Chapter 5). It has previously been shown that autocrine stimulation of the EGFR via TGF-α will only induce transformation if the EGFR is overexpressed (Di Marco *et al.*, 1987). Further analysis of the relationship between murine c-Kit expression and the induction of transformation via autocrine stimulation alone revealed that only 2 (NIH(mu*kit*).R67 and R68) of the 24 clones analysed produced colonies in the absence of added mSLF and these clones expressed high levels of c-*kit* mRNA and protein. However analysis of c-Kit expression levels in clones, NIH(mu*kit*).A-E, derived from colonies arising in the absence of added mSLF all expressed similar intermediate levels of c-Kit. Determination of *SLF* mRNA levels in NIH(mu*kit*).A-E and R67 and R68 cells indicated that the ability to grow in the absence of added factor did not correlate with the level of autocrine production. Thus, the molecular basis of transformation observed in the absence of added mSLF, and its dependence on autocrine factor remains unclear. It is possible that other events such as mutations within the c-Kit receptor (introduced during retroviral infection) or events not related to c-Kit were also involved in inducing transformation in the absence of exogenous mSLF. It should be noted though that these cells responded to mSLF when replated in the presence of factor.

The human c-Kit isoforms demonstrated discernible differences in their transforming potentials. The most potent isoform, c-Kit(GNNK-S+), was able to induce all the characteristics of transformation analysed in this study. In contrast c-Kit(GNNK+) was unable to induce tumourigenicity and the loss of the S_{715} also resulted in the loss of anchorage independent growth. The differences in the transforming potentials of the isoforms indicates that their alterations effect the functions of these receptors.

The more potent transforming capability of the c-Kit(GNNK-) isoform, further supported previous results demonstrating that the deletion of GNNK in the murine receptor was associated with its more enhanced signalling ability compared to the c-Kit(GNNK+) isoform (Reith *et al.*, 1991). Removal of GNNK from the human c-Kit receptor enabled cells expressing this isoform to attain a higher saturation density than NIH(GNNK+) cells expressing comparable receptor levels. The increase in proliferative capacity of c-Kit(GNNK-) expressing cells was also demonstrated by the 2-fold increase in hSLF-driven proliferation achieved by FD(GNNK-S+) compared to FD(GNNK+S+) cells. Also contributing to this may be the increased downregulation of the c-Kit(GNNK-) isoform compared to c-Kit(GNNK+) in FDC-P1 cells suggesting increased kinetics in the turnover of the former receptor. NIH(GNNK-S+) cells were also able to induce more efficient focus-formation than cells expressing comparable levels of the GNNK+ containing isoforms. However this enhancement was not evident in the anchorage independence assay which further demonstrates that these assays are analysing different aspects of c-Kit receptor function. C-Kit(GNNK-) was the only isoform able to induce tumourigenicity in *nude* mice, further emphasising the effect the GNNK- deletion had on enhancing the transforming activity of this isoform. It is interesting, that a similar deletion of 7-12 amino acids in the juxtamembrane region of the extracellular domain of the wild-type HER-2/*neu* receptor, also resulted in an enhanced transforming ability of the receptor (Siegel *et al.*, 1994). Similarly, insertion of a stretch of 40 amino acids within this region of the extracellular domain of the EGFR, resulted in the reduction of EGF binding and constitutive activation of the receptor, enabling cells expressing this mutant receptor to produce anchorage independent growth in the absence of factor (Sorokin *et al.*, 1995). These results in conjunction with those described

in this study for the c-Kit(GNNK-) isoform demonstrate the importance of this region to RTK function. Changes in this juxtamembrane region can possibly alter the conformation of these receptors resulting in long-range effects on ligand-binding and/or dimerization. This may also explain the reason for the differential inhibition of SLF-driven proliferation of FDC-P1 cells expressing thec-Kit(GNNK+/-) isoforms by a panel of anti-c-Kit mAbs (discussed in detail in Chapter 6).

The removal of S_{715} in the human c-Kit receptor reduced the transforming ability of c-Kit by preventing anchorage independent growth, although not altering its ability to induce focus-formation. As already mentioned above, it has recently been demonstrated that focus-formation and anchorage independent growth can be dissociated. Yu *et al.*, (1995) demonstrated that PLC-γ1 was important for the αPDGFR in inducing focus formation but not necessary for anchorage independent growth. Similarly the loss of S_{715} in the human c-Kit receptor may affect the binding of signalling molecules which may be essential for anchorage independent growth (discussed in more detail in Chapter 5). Alternatively, another event that may be affected by the removal of S_{715} involves serine phosphorylation. Serine residues are candidates of PKC-mediated phosphorylation, however S_{715} is not located within a PKC consensus sequence, and a recent study by Blume-Jensen *et al.*, (1995) did not depict S_{715} as a site of such phosphorylation. However, it is possible that other serine/threonine kinases may exist that could phosphorylate S_{715} and this needs to be addressed in the future. The loss of anchorage independent growth as a result of the loss of S_{715} was surprising since the murine c-Kit receptor which lacks the homologous serine residue was efficiently able to induce anchorage independent growth, although the murine construct also lacked the GNNK amino acids. At present the reason for these results are unclear and possibly may reflect slight divergence in signal transduction between the species and/or a result of the expression of the human c-Kit(GNNK+S-) receptor in a murine cell background. The human c-Kit(GNNK-S-) receptor, equivalent to the murine receptor, unfortunately did not express detectable protein (as discussed in more detail in Chapter 5) to allow for direct comparisons of their transforming abilities. Both the human

c-*kit(GNNK+/-S-)* cDNA constructs were sequenced, therefore it seems unlikely that the results obtained with these variants are artefactual.

Apart from the potential roles already described in this discussion and previous chapters it is possible that the various isoforms are also able to respond differentially to the soluble and membrane-bound isoforms of SLF. The availability of transfectant cell lines expressing the human isoforms of SLF in the laboratory will enable this to be easily addressed. With respect to cell adhesion, it has been shown that PI 3'-K plays a role in SLF-mediated cell adhesion of BMMC to fibronectin (Serve et al., 1994). As described in Chapter 5, the p85 binding site involving Y_{721} lays in close proximity to S_{715}. If removal of S_{715} affects the binding of p85 (currently being addressed in the laboratory) it is possible that the c-Kit(S-) may be impaired in its ability to bind to fibronectin upon SLF stimulation. Based on the results of the p85 experiments the latter will also be addressed. The deletion/insertion of GNNK may be involved in the production of soluble c-Kit receptors. The size of the soluble c-Kit product (~100 kd) (Yee *et al.*, 1994a; Brizzi *et al.*, 1994; Turner *et al.*, 1995) suggests that the proteolytic cleavage site is located within the extracellular domain within the juxtamembrane region. Since this is also the area in which GNNK is located it is possible that insertion/deletion of these amino acids may allow or prevent cleavage. The generation of cells independently expressing these isoforms in this study will enable culture supernatants to be analysed for the presence of soluble c-Kit in the future. The results obtained in Chapters 5 and 6 together suggest that the c-Kit isoforms individually differ in the recruitment of certain downstream signalling molecules/and or the strength and/or the duration of their activation and analysis of these events are the subject of future studies in the laboratory.

Investigation of the transforming potential of c-Kit was based on an earlier study in our laboratory, which suggested that high levels of c-Kit may play a role in leukaemogenesis (Ashman *et al.*, 1988) in a similar scenario to the overexpression of other RTK, HER-2/*neu* and EGF, in certain human cancers (Slamon *et al.*, 1987, 1989; Maa *et al.*, 1995 and references therein). Recent data from our group using more sensitive techniques has demonstrated thatc-*kit* RNA and c-Kit protein levels expressed by acute myeloid leukaemia (AML) blast cells, obtained from 70 patients, are generally lower than in normal CD34+

fractionated bone marrow cells. Of the blasts analysed, the approximate 20% that did express higher levels of c-*kit* (up to 3-fold) were generally of the undifferentiated phenotype (FAB M1 and M2) (Cole *et al.*, 1996). Therefore this higher level of expression may simply be due to the earlier stage of differentiation arrest of the blast cells. The reported poor remission rate associated with patients expressing high c-Kit levels on their blast cells (Ashman *et al.*, 1988) may be explained by recent data obtained in our laboratory, showing a correlation between c-Kit levels and functional multi-drug efflux activity (Sincock *et al.*, in preparation). It has recently been demonstrated that ectopic expression of murine c-Kit in the factor-dependent cell line, 32D, can render these cells leukaemogenic when injected into syngeneic mice. Although cells expressing the highest levels of c-Kit were able to induce more aggressive leukaemogenesis, it was shown that low expressing clones were also effective in inducing malignancy (Hu *et al.*, 1995). In the present study, it was shown that the levels of human c-Kit expression obtained in NIH/3T3 cells were lower or equivalent to those levels found on CD34+ fractionated bone marrow cells and were effective in inducing transformation. The studies described here therefore demonstrate that overexpression in relation to physiological c-Kit levels is not required to induce transformation of the indicator cell line NIH/3T3. In certain immature AML blast cells and the mouse model described above, the mere accumulation of cells constitutively expressing c-Kit may contribute to leukaemogenesis. The lack of maturation-linked downregulation of the c-Kit receptor in these systems may give the cells a competitive advantage, possibly due to an increase in SLF-mediated signalling and SLF-responsiveness of the cells as a result of their receptor density, as has been implicated in the model systems analysed in this current study.

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APPENDIX

Appendix 1: Reagents

A1.1: Reagents for APAAP technique

A1.1.1: Standard fixative: Mixed 47.5 ml acetone with 47.5 ml methanol and 5 ml formaldehyde.

A1.1.2: TBS: Consisted of 0.5 M Tris-HCl, pH 7.6 and 1.5 M NaCl in H_2O

A1.1.3: Substrate: Dissolved 20 mg napthol AS-mix phosphate free acid (Sigma, USA, Cat. No. L9756) in 2 ml dimethylformamide (BDH, Australia, Cat. No. 10322) and made up to 100 ml with 0.1 M Tris-HCl, pH 8.2. To this was added 100 µL 1 M levamisole (Sigma, USA, Cat. No. L9756). Added 10 mg Fast Red TR salt (Sigma, USA, Cat. No. F1500) /10 ml of the latter solution immediately prior to use. Dissolved and filtered through 3M Whatman paper onto slides standing in a coplin jar.

A1.1.4: Gill's haematoxylin: Reagents were added in the following order: 730 ml distilled H_2O, 250 ml ethylene glycol, 2.0 g Haematoxylin (anhydrous/monohydrate), 0.2 g sodium periodate ($NaIO_4$), 17.6 g aluminium sulphate ($Al_2(SO_4)_3.18H_2O$) and 20 ml glacial acetic acid. Allow solution to stir for 1 hour at RT. Stock matured with age allow to mature for 4 weeks prior to use. Staining times were adjusted according to the age of the stock.

A1.1.5: Scott's gentle alkaline solution: Dissolved 3.5 g $NaHCO_3$ and 20 mg Mg $SO_4.7H_2O$ in 1 litre H_2O.

A1.1.6: Glycerol-glycine: Added 1.4 g glycine to 100 ml H_2O and the pH was adjusted to 8.6 with NaOH. To 30 ml of this glycine buffer added 70 ml glycerol.

A1.2: Reagents for the Immune rosetting technique

A1.2.1: Alsever's solution: Dissolved 24.6 g glucose (BDH, Australia, Cat. No. 10117), 9.6 g tri-sodium citrate and 5.04 g NaCl in tissue culture grade Milli-Q-purified water to a final volume of 1 litre and pH to 6.1 with 1 M citric acid and made up to 1.2 litres. The solution was then filtered through a 0.22 μm bell filter (Millipore, USA, Cat. No. SVGB1010).

A1.2.2: Saline: Added 9 g NaCl to1 litre H_2O and the pH was adjusted to between 5-6 with O.1 M HCl or 1 M NaOH.

A1.4: Bacterial plates and media

A1.4.1: Ψa agar plates: Made up 5 g/ml Bacto-yeast extract (Difco, USA, Cat. No. 012-01-7), 20 g/l Bacto-tryptone (Difco, USA, Cat. No. 0127-01-7) 5 g/l $MgSO_4$ and adjusted the pH to 7.6 with KOH. To this was added 14 g/l Bacto-agar (Difco, USA, Cat. No. 0140-01) which was dissolved by autoclaving for 30 minutes at 130°C. This solution was allowed to cool slightly prior to pouring into petri dishes (Techno-Plas Cat. No. S-9014-520).

A1.4.2 : Ψb medium: Made as for Ψa agar plates in the absence of Bacto-agar.

A1.4.3: Tfb I Buffer: Made up 30 mM KOAc, 100 mM KCl, 10 mM $CaCl_2(2H_2O)$, 50 mM $MnCl_2$ ($4H_2O$), 15% (v/v) glycerol in H_2O and adjusted pH to 5.8 with acetic acid. This solution was filter-sterilised.

A1.4.4: Tfb II Buffer: Made up 10 mM MOPS (Sigma, USA, Cat. No. M-9381) (or PIPES (Sigma, USA, Cat. No. P-9291), 75 mM $CaCl_2(2H_2O)$, 10 mM KCl, 15%(v/v) glycerol in H_2O and adjusted pH to 6.5 with 1 M KOH and then filter-sterilised.

A1.4.5: Super Broth: Made up 3.2% w/v Bacto-tryptone, 2.0% Yeast extract, 0.5% NaCl in H_2O and adjusted pH to 7 - 7.2 prior to sterilising by autoclaving.

A1.4.6: S.O.C medium: S.O.C medium was made according to the recipe of BRL. Bacto-tryptone, Bacto-yeast extract, NaCl and KCl were dissolved in 97 ml distilled H_2O to give final concentrations of 2%, 0.5%, 10 mM and 2.5 mM, respectively, and autoclaved. $MgCl_2$ / $MgSO_4$ (10 mM each final) and glucose were added to a final concentration of 20 mM each. The medium was filter-sterilised through a 0.22 μm filter unit. The final pH was 7.0 ± 0.1.

A1.4.7: Luria Broth: Dissolved 10 g of Bacto-tryptone, 10 g NaCl and 5 g Bacto-yeast in H_2O and adjusted the pH to 7-7.2 with NaOH. This was made up to a final volume of 1 litre prior to autoclaving.

A1.4.8: Luria Broth Agar Plates: Melted 7.5 g Bacto-agar/500 ml Luria Broth by autoclaving. The agar was allowed to cool to approximately 56°C prior to the addition of 100 μg/ml ampicillin and then poured into petri dishes (Techno-Plas Cat. No. S-9014-520). Allow to set and then dry and store at 4°C.

A1.4.9: NaCl-TE saturated isopropanol: Added an equal volume of isopropanol to 5 M NaCl made in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 (Top layer = isopropanol).

A1.4.10: Sterile dialysis tubing: Boiled dialysis tubing once in 2% $NaHCO_3$, 1 mM EDTA for 10 minutes. Then washed tubing in sterile H_2O, 1 mM EDTA, pH 8.0. Tubing was stored in 1 mM EDTA, pH 8.0, 4°C.

A1.5 SEQUENCING REAGENTS

A1.5.1: 46% urea solution: Dissolved 460 g of urea (BDH, AnalR, Cat. No. 10290) in Milli-Q H_2O to a final volume of 1 litre. Added 20 - 30 g of Analytical Grade Mixed Resin AG 501-X8 (Biorad, Cat. No. 142-6424) and stirred for at least 30 minutes. Stored at RT in the presence of the resin which settled to the bottom of the bottle.

A1.5.2: 10x TBE: Dissolved 121 g of Tris base, 7.4 g of EDTA and 53.4 g boric acid in Milli-Q H_2O to a final volume of 1 litre. The pH was 8.3.

A1.5.3: 20% acrylamide solution: Dissolved 467 g urea in 500 ml of a 40% acrylamide solution (19:1, acrylamide : N, N'-methylene-bisacrylamide (Bio-Rad, Cat. No. 161-0144) and made up to a final volume of 1 litre in Milli-Q H_2O. To this was added 20-30 g of Analytical Grade Mixed Bed Resin AG 501-X8 and the mixture was stirred for at least 30 minutes. This was filtered through 3M Whatman paper and stored at RT in the dark.

A1.6: RNase-free treatment of reagents and apparti

A1.6.1: DEPC treatment of H_20 and glassware: H_2O used to make up solutions was treated with 0.1% v/v diethyl pyrocarbonate (DEPC) (Sigma, USA, Cat. No. D-5758) in a fume hood. The bottles containing the treated water were inverted several times to mix in the DEPC and let to stand at 37°C in an incubator overnight. The H_2O was then autoclaved to inactivate the DEPC and used to make RNA solutions. The empty DEPC-H_2O water bottles were then re-autoclaved and used to store RNA solutions.

Other glassware such as beakers and cylinders were made RNase-free by baking at 160 - 180°C overnight and then autoclaving.

A1.6.2: RNase-free treatment of ultra-turrax: The ultra turrax was soaked in 0.1% SDS, 0.1% DEPC (made fresh) for 5 minutes and then rinsed in 0.1% DEPC-treated H_2O to remove the SDS.

A1.6.3: RNase-free treatment of oligo-dT cellulose: The required amount of oligo-dT cellulose (0.025 g/ml) was washed in 0.5 M NaOH and then neutralised with several washes of DEPC-treated H_2O. The washed oligo-dT cellulose was then resuspended at 0.025 g/ml in Binding Buffer (refer to section 2.7.1 for recipe).

A1.7: mRNA analysis

A1.7.1: Sample buffer: Made up 100 µl 10 x Running Buffer (see A1.7.3(a)), 500 µl deionised formamide (see A1.7.5), 178 µl formaldehyde solution (35%)(BDH, AnalaR, Victoria, Australia. Cat. No. 10113) and 222 µl DEPC-treated H_2O.

A1.7.2: Loading buffer: Made up 0.25% bromophenol blue (BDH, England, Cat. No. 20015) 0.25% xylene cyanol FF (Sigma, NSW, Cat. No. X 4126) and 20% Ficoll (Type 400; Pharmacia, Sweden, Cat. No. 17-0400-01) in DEPC-treated H_2O.

A1.7.3: 1% RNA agarose gels: Dissolved 1 g of molecular biology grade RNase free agarose (IBI, Connecticut, USA. Cat. No. 70040) in 72 ml of DEPC-treated H_2O by microwaving. This was allowed to equilibrate to 56°C prior to adding 10 ml of 10 x Running buffer (see A1.7.3(a) below) and 18 ml of formaldehyde solution (35%)(2.2 M final) (BDH, AnalaR, Victoria, Australia. Cat. No. 10113). Gels were electrophoresed in 1 x Running buffer containing 2.2 M formaldehyde.

A1.7.3(a): 10 x Running Buffer: Made up 20 mM 3-[N-Morpholino]propane-sulfonic acid (MOPS) (Sigma, USA, Cat. No. M-1254), 1 mM EDTA, 5 mM NaAc pH 5.5. pH was adjusted to 7 with NaOH pellets.

A1.7.4: 10 x SSC: Dissolved 87.65 g of NaCl and 44.1 g of sodium citrate in H_2O. Adjusted pH to 7 with NaOH and made up to 1 litre.

A1.7.5: Prehybridisation Solution: The solution contained the following for 20 ml: 10 ml deionised formamide (see A1.7.5(a)), 5 ml 20 x SSC (2x A1.7.3), 2 ml 50 x Denhardt's (see A1.7.5(b)), 200 µl 10% SDS, 2 ml HEPES (of 100 mM, pH 7.1), 200 µl EDTA (of 100 mM, pH 7.5), 200 µl sodium pyrophosphate (of 200 mM, pH 7), 200 µl sheared and boiled salmon sperm DNA (of 10 mg/ml) and 30 µl tRNA (of 10mg/ml) (Gibco, Cat. No. 15401-011). A volume of 5 ml of prehybridisation solution was used to cover 100 cm^2 of membrane.

A1.7.5(a): Deionised formamide: Stirred formamide (BDH, AnalaR, Victoria, Australia, Cat. No. 103264R) with 20% w/v Analytical Grade Mixed Bed Resin AG 501-X8 (Bio-Rad Laboratories, Hercules, CA. Cat. No. 142-6424) for several hours. Filtered through 3 M Whatman, aliquoted and stored at -20^{o}C.

A1.7.5(b): 100 x Denhardt's reagent: Dissolved 10 g Ficoll Type 400 (Pharmacia, Sweden, Cat. No. 17-0400-01), 5 g polyvinylpyrrolidone (Sigma, NSW, Australia, Cat. No. P 6755), 5 g bovine serum albumin (BSA; Fraction V)(Cytosystems, Australia, Cat. No. 70-010-PF) in 500 ml H_2O.

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