THE ROLE OF EVI-1 IN CELLULAR TRANSFORMATION AND ITS BIOLOGICAL ACTIVITY IN PRIMARY BONE MARROW CELLS

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Abstract

The *EVI-1* proto-oncogene, which is frequently activated in murine retrovirally induced leukaemias and human acute myeloid leukaemias by non-random chromosomal translocations involving 3q26—where the *EVI-1* gene is located—encodes a transcriptional repressor. Repression is mediated by a 200 base pair proline-rich region designated Rp and also by a weaker second domain designated IR, which are both located between the two DNA binding domains (ZF1 and ZF2). Deletion mutagenesis studies, the results of which are reproduced in this thesis, reveal that the Rp, ZF1 and ZF2 domains are all required for optimal transformation of Rat1 fibroblasts. Moreover, it was demonstrated that several of these non-transforming evi-1 mutants, lacking either ZF1, Rp or both ZF1 and ZF2 (DNA binding defective mutants), could revert the transformed phenotype of evi-1-transformed Rat1 fibroblasts. Recent work has shown that evi-1 can bind the murine c-terminal binding protein 2 (mCtBP2) co-repressor and that evi-1 binds mCtBP2 through two conserved amino acid motifs residing in the Rp domain of the protein. The work described in this thesis has also shown that the integrity of these binding sites is crucial for evi-1-mediated repression and transformation in mammalian cells. Together these results suggested that ΔZF1 and ΔRp mutants with partially and fully intact DNA binding domains, respectively could inhibit transformation through a DNA binding competition for evi-1 binding sites. On the other hand, the DNA binding defective ΔZF12 mutants may sequester CtBP proteins or other factors necessary for FLevi-1 regulation and in turn inhibit the biological activity of the wild type protein.

In addition, until now no studies have addressed the significance of evi-1 functional domains for the biological activity of evi-1 in haematopoietic cells. Using retroviral infection of primary bone marrow cells it was shown that FLevi-1, the alternative evi-1 splice form Δ324, and ΔRp and ΔZF1 deletion mutants all blocked the production of erythroid and myeloid progenitors in an identical manner in methyl cellulose colony assays. The ΔZF2 and ΔZF12 mutants produced an intermediate phenotype. These results suggested that either distinct regions of the evi-1 protein are required for the transformation of fibroblasts and inhibition of haematopoietic cell colony formation or that the evi-1 proteins act in a dominant negative fashion over endogenous proteins required for haematopoiesis.

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Declaration

I declare that all the work in this thesis was performed personally unless otherwise acknowledged

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Abbreviations

Ac	.....acidic domain
ACF	.....ATP-utilising chromatin assembly and remodelling factor
AGM	.....aorta-gonad mesonephros
AML	.....Acute Myeloid Leukaemia
AML1/ETO	.....acute myeloid leukaemia 1/eight-twenty one fusion
Antp	.....Antennapedia
APL	.....Acute Promyelocytic Leukaemia
ATP	.....adenosine 5'-triphosphate
ATRA	.....all-trans retinoic acid
BCA	.....Bicinchoninic Acid Solution
BFU-E	.....burst-forming unit-erythroid
BFU-Meg	.....burst-forming unit/megakaryocyte
bHLH	.....basic helix-loop-helix
bp	.....base pairs
BSA	.....Bovine Serum Albumin
bZIP	.....Leucine Zippers or Factors
C/EBP	.....CCAAT/enhancer binding protein
CAF-1	.....chromatin assembly factor 1
CAT	.....choloroamphenicol transferase
CBF	.....core binding factor
CBP	.....CREB Binding Protein
CDKI's	.....cyclin-dependent kinase inhibitors
CDKs	.....cyclin dependent kinases
cDNA	.....complementary DNA
CFC	.....colony-forming cells
CFU-baso	.....colony forming unit-basophil
CFU-E	.....colony forming unit-erythroid
CFU-eos	.....colony forming unit-eosinophil
CFU-G	.....colony forming unit-granulocyte
CFU-GEMM	.....colony forming unit-granuolcyte/eythroid/monocyte/macrophage
CFU-GM	.....colony forming unit-granulocyte/macrophage
CFU-M	.....colony forming unit macrophage
CFU-Meg	.....colony forming unit-megakaryocyte
CIAP	.....calf intestinal alkaline phosphatase
CML-BC	.....chronic myelocytic leukaemia in blastic crisis
CTD	.....C-terminal domain
dCtBP	.....Drosophila C-terminal binding protein
DMEM	.....Dulbecco's Modified Eagles Medium
DMSO	.....dimethylsulphoxide
DNA	.....Deoxyribose Nucleic Acid
ECL	.....enhanced chemiluminescence
ECM	.....extracellular matrix
EDTA	.....ethylenediamine tetraacetic acid, disodium salt

EKLF	.....erythroid krüppel-like factor
Eng	.....Engrailed
EPO	.....erythropoietin
ES	.....embryonic stem
eve	.....Even Skipped 1
EVI-1	.....Ecotropic Viral Integration Site-1
FCS	.....feotal calf serm
FL	.....Full Length
FLevi-1	.....full length evi-1 (mouse)
FOG-1	.....Friend of GATA-1
G418	.....Geneticin
β-gal	.....β-galactosidase
G-CSF	.....granulocyte colony stimulating factor
GR	.....glucocorticoid receptor
GRE	.....glucocorticoid response element
HAT	.....hypoxanthine, aminopterin, and thymidine
HATs	.....histone acetyltransferases
HBS	.....hepes buffered saline
hCtBP1	.....Human C-terminal binding protein 1
HDAC	.....histone deacetylase
HT	.....hypoxanthine
HTH	.....Helix Turn Helix Motif
hTSHβ	.....human thyrotrophin
IL-3	.....interleukin 3
IR	.....intervening region
Itrp2	.....Inositol triphosphate type 2 receptor gene[2]
Kb	.....Kilo bases
kDa	.....kilodalton
KRAB	.....krüppel-associated box
LCR	.....locus control region
LMO2	.....LIM domain protein 2
LTR	.....Long Terminal Repeat
LTRSC	.....long term repopulating stem cells
mCtBP2	.....murine C-terminal Binding Protein 2
MDS	.....myelodysplastic syndrome
MDS-1	.....myelodysplastic syndrome 1
MLL	.....mixed lineage leukaemia protein
MoMLV	.....Moloney Murine Leukaemia Virus
MSV	.....Myeloproliferative Sarcoma Virus
MZF-1	.....myeloid zinc finger protein 1
NAP1	.....nucleosome assembly protein 1
NB	.....new born
NBCS	.....new born calf serum
NcoR	.....nuclear receptor corepressor
neo	.....Neomycin
Neo^r	.....Neomycin resistant
NPM	.....nucleophosmin
NuMA	.....nuclear matrix associated

OD .....optical density
PBS .....phosphate buffered saline
PCR .....polymerase chain reaction
PEBP2 .....polyoma enhancer binding protein 2
Ph .....Philadelphia chromosome
PLZF .....promyleocytic leukaemia zinc finger
PML .....promyelocytic leukaemia gene
PML/RAR .....promyelocytic leukaemia/retinoic acid receptor fusion
PPC .....pre-progenitor cells
PWM-SCCM .....Pokeweed Mitogen Stimulated Murine Spleen Conditioned Medium
RA .....refractory anaemia
RAEBT .....RA with excess of blasts in transformation
RAR .....retinoic acid receptor
RARE .....RA with excess of blasts
RARS .....refractory anaemia with ringed sideroblasts
RAT1FL .....Rat1 cells modified to constitutively express evi-1
RBC .....red blood cells
RI .....recombinant inbred
RNA pol II .....RNA polymerase II
RNA .....ribonucleic acid
Rp .....repressor domain
RXR .....retinoic acid receptor X
SA .....Splice Acceptor
SD .....Splice Donor
SDS-PAGE .....Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SIL .....SCL interrupting locus
SMRT .....silencing mediator of retinoid and thyroid hormone receptor
SRB .....“suppressor” of RNA polymerase B
TAFs .....TBP-associated factors
TEMED .....N,N,N’,N’-tetramethylethylenediamine
T-ALL .....T cell acute lymphoblastic leukaemia
TBP .....TATA-Binding Protein
tk .....thymidine kinase
TLC .....thin layer chromatography
TR .....thyroid hormone receptor
Tris .....tris-[hydroxylmethyl]aminomethane
UV .....ultra-violet
VDRE .....vitamin D3 response element
WT-1 .....Wilms Tumour Suppressor Gene
WT1 .....Wilms Tumour 1
w/v .....weight for volume
w/w .....weight for weight
zeo .....Zeocin
Zeo^r .....Zeocin resistant
ZF1 .....First DNA binding domain of Evi-1
ZF12 .....Both DNA binding domains of Evi-1
ZF2 .....Second DNA binding domain of Evi-1

Abbreviations for amino acids:

Amino acid	Three-letter abbreviation	One letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Asparagine or Aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Gultamine or Glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	X

Throughout this thesis, the names of human proteins are presented in normal text capital letters (e.g. EVI-1) and mouse proteins in normal small case lettering (e.g. evi-1). Human genes are presented in italic text capital letters (e.g. *EVI-1*) and mouse genes in small case italics (e.g. *evi-1*).

Chapter 1: Introduction

1.1 Eukaryotic gene transcription: An Intertwined Network of Chromatin Remodelling Machines and Transcription Factors

A cell must regulate the transcription of nearly all its genes and therefore it stands to reason that a high proportion—an estimated 10%—of a cell's genes are exclusively set aside for proteins involved in transcriptional regulation. The appropriate genes must be transcribed and this requires an open chromatin conformation together with the required transcription factors. However, after transcription initiation many other modes of regulation can be imposed. These include transcript elongation, stability and polyadenylation and each of these can be regulated in a cell-specific manner. Translation itself and post-translational protein modifications provide an additional layer of complexity whereby protein production can also be modulated.

Advances within the field of gene regulation are fast unfolding and studies continue to reveal a multitude of regulatory mechanisms controlling differential gene expression in particular cell types. The following sections will focus on transcription and aspects of its regulation including new concepts that illuminate the importance of histone-DNA liaisons within this process.

1.1.1 Unravelling Chromatin Precedes the Initiation of Transcription

Over 2m of DNA is extensively folded into a human cell in such a way that it squeezes into a nucleus of around 20μm diameter. The primary level of compaction of eukaryotic DNA is the formation of linear arrays of nucleosome core particles. A nucleosome consists of around 140 base pairs of DNA wrapped 1.8 times around a complex of eight histones two each of H2A, H2B, H3 and H4. This initial arrangement, referred to as the "beads-on-a-string" structure, is then further folded into a solenoid arrangement of at least 30nm in diameter. Looped fibres of around 100-200nm predominate the bulk chromatin structure of the interphase nucleus—a cell cycle period in which the genome must be accessible for transcription, replication, repair and recombination processes. It is this packaging of the eukaryotic genome into chromatin that permits dynamic unfolding of specific loci so that DNA sequences are accessible to enzymes that "read" the cells genetic material. (Reviewed in Kornberg and Lorch, 1999, Koshland and Strunnikov, 1996, Lodish et al., 1995).

Coactivator multiprotein complexes with histone acetylase transferase (HAT) activity can stimulate transcription at several levels including the stimulation of the pre-initiation complex and remodelling of chromatin (Reviewed in Kornberg and Lorch, 1999, Davie and Spencer, 1999, Kuo and Allis, 1998 and Davie and Chadee, 1998). One way of making chromatin accessible appears to be regulated by acetylation of free amino acid groups of lysine at the N-terminal end of positively charged histone molecules by

coactivator molecules: this results in a decreased net positive charge, a lower affinity for the DNA phosphate backbone and stimulation of transcription. Conversely, a histone deacetylase (HDAC) corepressor complex can deacetylate histones and transcription factors to prevent the formation of the pre-initiation complex (Reviewed in Hui and Bird, 2000, Kornberg and Lorch, 1999, Kuo and Allis, 1998, Davie and Chadee, 1998, and Davie and Spencer, 1999): the net result is a repression of transcription. Current mechanisms concerning chromatin remodelling are discussed in section 1.1.3.2.4, below. Regardless of the exact mechanism, it is widely accepted that a relaxation or activation of chromatin is imperative for the onset—and maintenance—of gene transcription.

1.1.2 Initiation of Transcription—Assembly of the General Transcription Factors

The regulation of transcription initiation is a major point of control for gene expression in multicellular organisms ranging from invertebrates to mammals (Reviewed in Halle and Meisterernst, 1996). The initiation of transcription by RNA polymerase II (RNA pol II) requires stable assembly of the polymerase at the promoter as a large pre-initiation complex. The components of the complex have been elucidated over many years and include basal or general transcription factors (GTFs): TFIIA, TFIIB, TFIID, TFIIF, TFIIE and TFIIH. Of these TFIID and TFIIH are also comprised of many subunits and both contain independent activities. TFIID consists of the TATA-Binding Protein (TBP) plus TBP-associated factors (TAFs) and specifically binds DNA, and TFIIH includes both

kinase and helicase activity. It is now recognised that these GTFs gain access to the promoter in a highly regulated and defined order.

In most eukaryotic core promoters a TATA box element is located approximately 25 base pairs (bp) upstream of the transcription initiation start site which is recognised by TATA-binding protein (TBP). By binding the TATA box, TBP initiates GTF assembly. The TAFs then bind to TBP, creating TFIID. GTF assembly continues with the recruitment of TFIIA, TFIIB and the remaining GTFs. TFIIB facilitates the association of the pre-assembled RNA pol II. Subsequently a large RNA pol II complex forms—consisting of the multi-subunit polymerase II in a large conglomerate with "mediator" (which consists of "suppressor" of RNA polymerase B (SRB) proteins and coactivator proteins—distinct from the basal transcription factors) in addition to some of the basal transcription factors. Initiation of transcription (formation of the first phosphodiester bond in the RNA) leads to a transition of the polymerase, still complexed with a subset of basal transcription factors into an early elongation state. A subsequent transition, sometimes called promoter clearance results in the stable elongation complex of polymerase, DNA, RNA and associated elongation factors. A great deal of phosphorylation is observed in the C-terminal domain (CTD) of RNA Pol II in the elongation complex. Once elongation has been terminated, RNA pol II detaches from DNA and, before it can re-enter initiation, the CTD must be dephosphorylated Although the presence of a core promoter sequence and the assembly of basal transcription apparatus is sufficient for transcription initiation of a specific gene—albeit minimal levels—upstream factors are required to modify transcriptional rates.

1.1.3 Regulation of Transcription

1.1.3.1 Regulatory Elements

Enhancers

Enhancers are positive regulatory elements that serve to increase the basal level of transcription, which is initiated through the core promoter elements. Their functions, unlike those of the core promoter, are independent of their orientation and distance from the gene they regulate (Blackwood and Kadonaga, 1998). Indeed enhancer elements can activate transcription at considerable distances. They often contain elements recognised by ubiquitous transcription factors and elements recognised by tissue-specific transcription factors, thereby providing a basis for tissue-specific gene expression. Enhancer motifs have been identified that are required for transcriptional induction by various agents. Among these are the glucocorticoid response element (GRE), which binds the glucocorticoid receptor (GR), the serum responsive element of the c-fos gene and the heat shock response elements of the heat shock genes (reviewed in Atichson,1988).

Silencers (Silencer Elements)

Silencers, as the name suggests, are regulatory sequences that inhibit transcriptional activity of specific genes. A silencer is a short, specific sequence of nucleotides that tend to be located 5' upstream of the promoter region of a gene. These sequences recruit

transcription factor proteins to the promoter, which in turn perform specific functions to ultimately down regulate gene expression. It has become evident that many different types of silencers are involved in the regulation of many aspects of gene regulation—such as cytoplasmic retention of transcription factors, activity of transcriptional activators, chromatin structure, intron splicing and GTF assembly (Clark and Docherty, 1993)—to ultimately down regulate gene expression. One classical silencer is the AT-rich element found in human thyrotrophin (*hTSH*β) gene promoter, which consists of multiple binding sites for the ubiquitous POU homeoprotein, OCT-1 (Kim et al., 1996).

1.1.3.2 Transcription Factors

Transcription factors are modular proteins that recognise short conserved nucleotide sequences involved in regulating the transcription of various genes. The region of DNA-protein binding is typically characterised by around 20 amino acid/nucleotide contacts, which ensures strong and specific binding. In human and other eukaryotic transcription factors, several distinct functions can often be identified and located in different parts of the protein: (1) DNA binding domains, (2) dimerisation domains, (3) activation domains and (4) repressor domains. Transcription factors, with often quite different specificities, are characterised into several "families" by common structural motifs. Indeed, identification of these domains has provided some insight into how these factors can bind DNA, contact a variety cellular target proteins and how such complex interactions might influence gene expression and ultimately cell-specific proliferation/differentiation.

Some of the major structural domains implicated in eukaryotic gene regulation are described below.

1.1.3.2.1Conserved Families of DNA-Binding/Protein Interaction Domains

Leucine Zippers or bZIP Factors

The leucine zipper is a helical stretch of amino acids rich in leucine residues, (typically one every 7 amino acids) which was first identified as a dimerisation domain in the CCAAT/enhancer binding protein (C/EBP) (Landshultz et al., 1988). Each monomer unit consists of an amphipathic α-helix of hydrophobic amino acids facing one way and polar groups facing the other way. Two α-helices of individual monomers join over a short distance to form a coiled coil structure (O'Shea et al., 1991) with predominant interactions occurring between hydrophobic amino acids of the individual monomers. The region beyond these interactions separates, so that the overall dimer is Y-shaped, allowing the dimer to grip the double helix like a clothes peg through basic DNA binding motifs, hence the term bZIP factors. Leucine zippers are also able to occasionally form heterodimers as well as homodimers, which provide essential combinatorial control mechanisms for gene regulation. The oncoproteins FOS and JUN (Glover and Harrison, 1995) and the yeast regulatory protein, GCN4 (O'Shea, 1991) belong to the large family of bzip transcriptional modulators.

Helix Loop Helix (HLH) Motif

The (HLH) motif consists of two α-helices, one short and one long, connected by a flexible loop—that enables the two helices to lie in planes that are parallel to each other. The HLH motif mediates both DNA binding and protein dimer formation (Murre et al., 1989) and it permits occasional heterodimer formation (Henthorn et al., 1990). Heterodimers can form between a full length HLH protein and a truncated HLH protein (which lacks the entire region of the α-helix necessary to bind DNA tightly). Therefore, these heterodimers are thought to inactivate specific gene regulatory proteins. Members of the HLH family include MAX, E47 and MYOD (reviewed in Kohn et al., 1997).

Helix Turn Helix (HTH) Motif

The homeobox-containing homeotic genes were originally identified in *Drosophila* with the proteins they encode being critical for regulating development (De Robertis et al., 1990). Homeobox-containing proteins have been found in all species from yeast to humans and homeodomains are remarkably conserved across species (reviewed in Patikoglou and Burley, 1997), although the remainder of the protein structure may be divergent. The HTH motif is common to homeodomains. This motif is characterised by two short α-helices separated by a short amino acid sequence, which creates a β-turn, so that the two helices are orientated on different planes. The more C-terminal helix acts as the specific recognition helix as it fits into the major groove of DNA. HTH proteins include the *Drosophila* homeodomain proteins *Antennapedia (Antp)* and *Engrailed(Eng)*, the yeast protein MATα2 and the mammalian protein C-MYB (reviewed in Patikoglou and Burley, 1997).

Zinc Fingers

Zinc finger protein modules are highly abundant across all species from yeast to mammalian genomes. The zinc finger motif is co-ordinated with a zinc ion by four conserved amino acids to form a loop or "finger", a structure often arranged in sequential arrays. Zinc fingers can be divided into different sub-groups by the nature and spacing of their zinc chelating residues. The two most common forms are the classical CCHH, TFIIIA (Miller et al., 1985) or C_2H_2 zinc fingers (characterised by the binding of a zinc ion by two conserved cysteine residues and two conserved histidine residues) and the GATA-like CCCC (Omichinski et al., 1993) or C_2C_2 fingers (consisting of four conserved cysteine residues). However, common to both forms is an α-helix and a β-sheet folded around a zinc ion and it is the α-helix that makes contacts with the major groove of DNA (Parraga et al., 1988 and Omichinski et al., 1993). Recent studies have also uncovered the role of zinc finger motifs in mediating protein-protein interactions (Tsang et al., 1997 and Merika and Orkin, 1995). Examples of zinc finger proteins include the eukaryotic GTF, TFIIA (Miller et al., 1995), Ying Yang 1 (YY1) (Shi et al., 1996) and the *Drosophila* protein, *u-shaped* (Haenlin et al, 1997).

1.1.3.2.2Trans-Activation Domains

Many of the promoter-specific regulatory proteins are sequence-specific DNA binding proteins capable of functioning as transcriptional activators (Hope and Struhl, 1986 and Keegan et al., 1986). Activation domains are large, often map to multiple regions within a protein and have been loosely classified as to whether they are rich in glutamine, proline or acidic amino acids (Seipel et al., 1992). The serum response elements of the steroid hormone receptor superfamily represent another class of activation domain (Treisman, 1992). Indeed, proteins belonging to these different classes modulate transcriptional activation in very different ways: (1) "Proximal" activation domains, typified by the glutamine-rich domains of SP1, OCT1 and OCT2, only stimulate transcription from a position close to the TATA box, usually in response to a remote enhancer (Seipel et al., 1992) (2) Acidic domains, exemplified by the yeast DNA binding protein GAL4 and herpes simplex virus VP16, stimulate transcription from both remote and proximal promoter positions (Seipel et al., 1992). (3) Proline-rich activation domains found in AP-2 and CTF/NF-1 demonstrate considerable proximal but little remote promoter activity (Seipel et al., 1992).

Most activators are regarded as "promiscuous" and are thus able make direct physical interactions with sequence-specific transcription factors or with the GTFs. For example the HSV protein VP16 binds sequence-specific OCT-1 and the GTFs TBP, TFIID, $TAFII_{31}$, TFIIB, TFIIA and TFIIH (reviewed in Flint and Shenk, 1997). Activators may enhance the formation of the pre-initiation complex by a "direct" mechanism (which

involves interaction with free GTFs and/or a holoenzyme complex that recruits these components to the core promoter) or by an "indirect" mechanism (involving interactions with, and modifications of, a pre-existing TFIID-core promoter complex that in turn mediates the recruitment of the other components). Co-activator proteins are cofactors that are required to recruit the transcriptional machinery—and ultimately RNA pol II—to transcribe target genes. Recent studies have also shown that a number of coactivators regulate the remodelling of chromatin (reviewed in Xu et al., 1999 and Torchia et al., 1998). It appears that coactivators can recruit histone acetyltransferases (HATs), which catalyse a process that destabilises histone-DNA contacts by histone acetylation, thereby allowing the binding of other transcription factors.

1.1.3.2.3Repressor Domains

DNA binding transcriptional repressors act by a variety of mechanisms including direct interactions with activators or GTFs, competition with activators or GTFs for access to the DNA or the recruitment of corepressors with intrinsic repression activity. Each repressor generally contains a small repression domain. Similar to activation domains, repressor regions are characterised according to the primary amino acid content. However, these inhibitory domains are often small and discreet, compared to activation domains, and found in defined regions of proteins (reviewed in Hanna-Rose and Hansen, 1996). Some repressor domains are alanine-rich like the N-terminal repression motif in *Drosophila* gene *krüppel* (Licht, 1994). Several others are proline-rich such as the repressor domains belonging to the oncoprotein, Ecotropic Viral Integration Site-1 (EVI-

1) (Bartholomew et al., 1997) and suppressor protein, Wilms Tumour 1 (WT1) (Saha et al., 1993). In addition, high charge represents a common feature of repressor regions: an example of this is a potent repressor region named *krüppel*-associated box (KRAB) (Witzgall, 1994). Much larger, charged and hydrophobic residue repressor regions are present in the well-characterised thyroid hormone receptor (TR) and the retinoic acid receptor α (RARα) (Baniahmad et al., 1992). Several unique repressor motifs have also been described (Wang et al, 1995 and Gashler et al., 1990). Potential mechanisms for the negative regulation of transcription by repressors are discussed below.

Four major mechanisms of transcriptional repressor function are hypothesised:
(1) Repressors may directly compete with activators for binding to a common target DNA or (2) may bind simultaneously with activators but may "quench" their functions, possibly by masking the activation domain. (3) Repressors may bind DNA and interact with the general transcription machinery inhibiting a transcriptionally competent state. (4) Repressors may also recruit corepressors. Interactions of repressors with such targets have been described and suggest a direct involvement in transcriptional repression.

Competitive binding

A repressor may bind directly to DNA and mask a transcriptional activation domain. For instance, the vitamin D3 response element (VDRE) of the bone protein, osteocalcin mediates regulation by vitamin A and vitamin D3 receptors and surprisingly, the oncogene *jun-fos*. As such, *jun-fos* is able to suppress basal levels of the osteocalcin

gene and down-regulate hormonal induction by vitamins A and D3 *in vitro* (Schüle et al., 1990).

Repressor interactions with activator or coactivator proteins

Repressors that can interact with activator or coactivator proteins are predicted to be able to inhibit only certain promoters because they interfere with the activity of one or more activators that specifically bind that promoter. A recent study using *in vitro* assays has shown that the haematopoietic transcription factor, GATA-1 can negatively regulate myeloid differentiation by directly interacting with the PU.1 transactivator—arguably the most crucial factor known for the development of myeloid progenitors *in vivo* (Nerlov et al., 2000).

Repressor interactions with basal transcription factors

Repressors that react with basal transcription factors are predicted to be able to repress a minimal RNA pol II promoter which contains only a TATA box or initiator element (Fondell et al., 1993). Several models have been put forward to describe how this might work. For instance, the repressor may interfere with the proper assembly of any one of the general transcription factors to the pre-initiation complex; or the repressor could interact with the general transcription machinery preventing an isomerisation or disassembly step—resulting in a kind of "frozen" assembly. Another possibility would be that a "saboteur" factor could be loaded onto the general assembly by the repressor. This could perhaps act through either of these aforementioned mechanisms. The repressor domain of the *Drosophila* Even Skipped (eve) homoeodomain protein can interact

directly with TBP *in vivo* and *in vitro* (Um et al., 1995). More recently, the repressor activity of EVE was demonstrated to be evolutionarily conserved when EVE was found to be a potent repressor of several promoter-types in mammalian cells. Moreover these studies confirmed that EVE specifically inhibits the pre-initiation complex by preventing the assembly of TFIID (McKay et al., 1999).

Repressor interactions with corepressor proteins

Corepressors are proteins that bridge the interaction of the repressor with its ultimate target. Several sequence-specific repressor proteins have been identified, which are essential for *Drosophila* development (Rivera-Pomar and Jackle, 1996). These repressors fall into two categories: (1) Short-range repressors that work over distances of less than 100 base pairs to inhibit the core promoter or quench upstream activators and (2) Long range repressors that work over distances of 1Kb or more to repress transcription. Although the exact mechanisms are unknown, it appears that two evolutionary conserved corepressor proteins, Groucho (Fisher and Caudy, 1998) and dCtBP (*Drosophila* C-terminal binding protein) (Nibu et al., 1998)) are essential for the long and short range modes of repression, respectively. CtBP factors are conserved across species and mammalian homologues have been identified and characterised (Schaeper et al., 1995 (human) Turner and Crossley et al., 1998 (mouse)).

In the last 5 years, a number of mammalian nuclear hormone receptor interacting proteins have also been isolated including nuclear receptor corepressor (NcoR) (Horlein et. al., 1995 and Kurokawa et al, 1995B) and its homologue silencing mediator of retinoid and

thyroid hormone receptor (SMRT) (Chen and Evans, 1995). These corepressors interact with several unliganded receptors including the retinoic acid receptor (RAR) and thyroid hormone receptor (TR). These proteins contain transferable repressor regions and it is believed that they function in a multicomponent corepressor complex containing histone deacetylase (HDAC) activity and the re-establishment of a repressive chromatin state (Hienzel et al., 1997).

1.3.2.4Histones - Repressor and Activator functions

Histone modification facilitates nucleosome assembly and remodelling through acetylation or deacetylation of histone "tails" at the N-terminus or by perturbation of DNA-histone contacts. Several key factors have now been identified with intrinsic chromatin remodelling activity, which has been linked to both gene regulation and the initiation of transcription, amongst other processes: 1) Chromatin assembly factors (reviewed in Kornberg and Lorch, 1999). Chromatin assembly is fundamental for duplication and maintenance of the genome. The core chromatin assembly machinery required for ATP-dependent assembly of periodic nucleosomal arrays comprise a protein complex called ATP-utilising chromatin assembly and remodelling factor (or ACF) with a core histone chaperone such as chromatin assembly factor 1 (CAF-1) or nucleosome assembly protein 1 (NAP1) (Ito et al., 1997A and Ito et al, 1997B). 2) Histone acetylases and deacetylases. When the core histones are acetylated a neutralisation of charge on lysine residues causes a reduction in the affinity of histone-DNA interactions leading to increased access of transcription factors to the repressed chromatin template. Indeed,

there is a positive correlation between the extent of core histone acetylation and gene activity (reviewed in Kouzarides, 1999). Histone aceytltransferases (HATs) include the HAT1 protein, GCN5 protein, the TAFII250 subunit of TFIID, CBP/p300, P/CAF, and the SRC-1 family of coactivators (reviewed in Torchia et al., 1998, Kuo and Allis, 1998). Conversely, acetyl groups are removed from histones by specific histone deacetylase (HDAC) enzymes. Histone deacetlylation is associated with transcriptional repression and HDACs include HDAC1, HDAC2 and RpAp48 (reviewed in Torchia et al., 1998, Kuo and Allis, 1998 and Ng and Bird, 2000). 3) DNA methylation (reviewed in Ng and Bird , 1999). Methylation of cytosines in a DNA recognition element can block the binding of sequence-specific trans-acting proteins (Hsieh, 1999). Other studies suggest that methylation of cytosine residues at CpG sites may provide direct binding sites for certain DNA binding activators or repressors. Indeed, the MeCP2 chromosomal protein binds specifically to methylated DNA, via its methyl CpG binding domain, and represses transcription by displacing histone H1(Nan et al., 1997). Interestingly, recent studies have shown that MeCP2 can also bind to components of the mSin3A/HDAC complex. Moreover the repressive activities of MeCP2 repressor domain can be alleviated upon addition of Trichostatin A-specific HDAC inhibitor (Nan et al., 1998). 4) Chromatin remodelling machines. These molecular machines include multiprotein complexes in yeast (SWI/SNF and RSC), in Drosophila (NURF, BRM, CHRAC and ACF complexes) and in mammals (BRG1- or hbrm- associated complexes) (reviewed in Kornberg and Lorch, 1999). Each of these complexes contain a ribonucleoside triphosphate (NTP) binding subunit which is thought to act as a processive ATP-binding DNA-translocating motor that disrupts histone-DNA interactions.

Emerging studies provide evidence for an interlaced interplay amongst these mechanisms and transcription factors to facilitate cellular processes in the context of chromatin (Kadonaga, 1998 and Bestor, 1998). At present, histone acetylation and methylation represent the best-understood mechanisms of chromatin remodelling. However, many fundamental questions concerning this new exciting field remain unanswered such as what mechanisms direct these remodelling complexes to chromatin, what controls their range of action and what underlies their diversity. In addition, considering all DNA templated processes (transcription, replication, recombination, repair and segregation) deal with DNA packaged into chromatin, future studies elucidating conceptual links between these nuclear processes are eagerly awaited.

1.2 Normal Haematopoiesis

1.2.1 The Origins of Haematopoiesis

It has been known for many years that the establishment of the haematopoietic system occurs at specific sites in the embryo through a succession of developmental programs, which result in the production of precursor colony-forming cells (CFC) that in turn differentiate into all mature blood cell types entering the circulation (reviewed in Metcalf, 1999).

Today, the ontogenic source of definitive (adult) haematopoietic stem cells in the developing foetus is a controversial issue—with developmental biologists debating over an intra- or extraembryonic origin for blood stem cells. Evidence from some studies indicates that an intra-embryonic site called the aorta-gonad mesonephros (AGM) is the sole site of origin of definitive haematopoiesis (Medvinsky et al., 1993, Medvinsky and Dzierzak, 1996). On the other hand, more recent work suggested that haematopoietic events in the mouse embryo are initiated in the yolk sac and the AGM (Dzierak et al., 1998). The most recent studies in this field propose a new paradigm for blood cell production in the developing foetus where the yolk sac is the exclusive site of primitive (embryonic) haematopoiesis. However, definitive haematopoiesis occurs in both the yolk-sac and the AGM (Palis et al., 1999) (Figure 1). It is proposed that the yolk sac provides two important functions within the developing mouse embryo. Firstly, between embryonic day 7.0 (E7.0) and E9.0 in the extraembryonic mesoderm, where blood islands are formed (Haar and Ackerman, 1971 cited in Palis et al., 1999). This primitive, transient wave of cells are incredibly short lived and sustain the survival and growth of the embryo until liver rudiment colonisation at E12 (Copp, 1995). Secondly, definitive haematopoietic progenitors are synthesised in the yolk sac between E8.5 and E10, possibly derived from newborn repopulating cells found in this tissue (Yoder et al., 1997), which in turn rapidly enter circulation. It is postulated that progenitors subsequently seed the liver at E9.5 to initiate intraembryonic haematopoiesis. As development progresses colony forming unit-spleen (CFU-S), multipotential progenitors and long term repopulating stem cells, derived independently in the AGM region at E9 to late E10 (Medvinsky and Dzierak, 1996), seed the liver at E11 and ultimately replace the yolk sac-

derived circulating cells. These AGM-derived cells are found thereafter in the bloodstream of the embryo proper. Later this haematopoietic activity shifts to the spleen, and thereafter to the bone marrow.

Future studies must clearly define the relationship and the exact origins of the primitive and definitive haematopoietic progeny.

1.2.2 Haematopoiesis in the Adult

After birth definitive haematopoiesis occurs in the bone marrow (also the spleen in mice). The principle functional obligation of the bone marrow is to supply circulating blood with mature blood cells during steady-state conditions and during periods of increased demands. In the normal adult the daily production and export of cells within the marrow amount to approximately 2.5 billion red cells, 2.5 billion platelets and 1 billion graulocytes per kilogram of body weight (Erslev and Lichtman, 1990). Blood cells are produced within a specialised environment known as the haematopoietic microenvironment, consisting of different cell types (fibroblasts, macrophages, endothelial cells and adipocytes) and their products (cytokines and extracellular matrix (ECM)) (reviewed in Whetton and Spooncer, 1998). All mature blood cells ultimately arise from haematopoietic stem cells, which are pluripotent cells capable of self-renewal, maintenance, and differentiation into all haematopoietic lineages (Keller et al., 1985). These stem cells give rise to progenitors that lack the capacity for self-renewal. These progenitors in turn give rise to more mature precursor cells, which are committed to a

specific haematopoietic lineage and are morphologically recognisable (Metcalf, 1984 cited in Metcalf, 1998). Cultured *in vitro*, some progenitor cells are bipotential or multipotential (e.g. erythroid/megakaryocte (BFU-Meg), granulocyte/macrophage (CFU-GM) or granulocyte/erythroid/megakarocyte/macrophage cells (CFU-GEMM). However, most of these committed progenitors form colonies composed of a single lineage (e.g. erythroid (BFU-E), granulocyte (CFU-G), macrophage (CFU-M), eosinophil (CFU-Eos), basophil (CFU-Baso), or megakaryocyte (CFU-Meg) cells. It is these precursors that give rise to the mature, terminally differentiated blood cells found in the circulation. The generally accepted hierarchical scheme of haematopoiesis is shown in Figure 2. However, this rigid fate map is likely to be a deceptively simplified scenario. Indeed, although it is reasonable to assume that multilineage progenitors are partially descendant from either bipotential or unipotential progenitors, the relative frequencies and varying proliferative potential of these different progenitor cells suggests that not all single lineage progenitors are necessarily the descendants of bipotential or multipotential progenitor cells. Furthermore, recent studies have provided evidence for the existence of a distinct class of transit cells within this scheme called pre-progenitor cells (PPC) (Metcalf, 1998 and reviewed in Metcalf, 1999). These cells are believed to be the progeny of stem cells and the ancestors of lineage committed progenitor cells. However these studies suggested that not all of the lineage committed progenitor cells are the progeny of pre-progenitor cells since some are formed directly by pluripotent stem cells. Other studies utilising new methods that facilitate the isolation of multipotential cells have provided an opportunity to interrogate the mechanisms of hierarchical differentiation. These studies have lead to the notion that selective gene activation is a

multistep process that may ultimately depend on chromatin changes (Felsenfeld et al., 1996 and Flickinger, 1999). Another consideration is the mode of cytokine action: it is not clear whether these growth factors instruct lineage selection or merely provide a permissive environment for stochastic programs of differentiation. In summary, a degree of hierarchical structure within haematopoiesis is a certainty but intermediary cell states must be fully scrutinised to gain a more realistic model of this process.

One common pathway of haematopoietic lineage restriction involves the formation of stable transcription complexes that instigate exclusive programs of gene expression. Indeed, it is believed that these processes governing cell differentiation rely on the combinatorial action of both lineage-specific and ubiquitous transcription factors. Exactly how the expression of transcription factors is initiated remains unclear. Possibilities include orchestration of factors by stochastic expression of key lineage-affiliated regulators or a finely tuned response to instructed expression. Whatever the mechanism, the past decade has proved a fruitful time for the identification and characterisation of these key regulators of haematopoietic development, some of which will be discussed in section 1.2.2.1 that follows.

1.2.2.1 Nuclear Regulators in Haematopoiesis

Our interests concerning haematopoietic development will focus on the erythroid and myeloid lineages in later chapters of this thesis. Therefore the regulation of lymphopoiesis and megakaryopoiesis will not be discussed here. However, these

biological processes were reviewed recently in Henderson and Calame, 1998 (B-cell development), Kuo and Leiden, 1999 (T-cell development) and Baatout, 1998 (megakaryopoiesis). The principle factors involved in erythroid and myeloid regulation are addressed below.

1.2.2.1.1Transcriptional Control of Erythropoiesis

Studies examining cis-elements and globin gene control in erythroid cells defined distant regulatory sequences known as the locus control regions (or LCRs), which act over large distances within the globin loci. Interestingly, three motifs are common to the LCRs and promoters of all erythroid-specific genes examined-to-date including NF-E2 and GATA (Orkin, 1995 and Baron, 1997).

The *NF-E2* gene product is a bZIP transcription factor, which also contains a dimerisation domain. NF-E2 is an obligate heterodimer of a haematopoietic-specific p45 subunit and a ubiquitous p18 subunit that belongs to the MAF family of proteins (Blank et al., 1997 and Francastel et al., 1997). Several earlier studies provided evidence for NF-E2 as the major enhancer function of β- globin gene expression (Talbot et al., 1990, Jarman et al., 1991, Shivadasani and Orkin, 1996). Absence of p45 nf-e2 in mice results in mild, albeit consistent, red blood cell abnormalities, including hypochromia, anisovytosis and reticulocytosis (Shivadasani and Orkin, 1995). NF-E2 is also expressed in megakaryocytes and mast cells (Andrews et al., 1993). Others have implicated phosphorylation in the regulation of *NF-E2* transcriptional activity during erythroid differentiation (Nagai et al., 1998). Recently, the p45 subunit of NF-E2 has been

recognised to mediate protein-protein interactions: p45 can interact with key regulators of chromatin modification and transcriptional activation namely, the CREB Binding Protein (CBP) and $TAF_{II}130$ (Cheng et al., 1997 and Amrolia et al., 1997 respectively).

GATA-1, the founding member of the *GATA* factor family, is one of the most extensively studied erythroid transcription factors. It is a member of a highly conserved family of transcription factors, which bind the conserved motif GATA, present in all erythroid-specific promoter regions investigated so far. Therefore, it is considered that *GATA-1* is involved in regulating most, if not all, erythroid specific genes. Gene targeting experiments reveal that a loss of *GATA-1* results in a developmental arrest at the proerythroblast stage and apoptosis (Penvy et al., 1995, Weiss et al., 1994 and Weiss and Orkin. 1995), which results in embryonic lethality by E11.5 (Fujiwara et al., 1996). Others have demonstrated that the GATA-1 protein consists of an N-terminal activation domain (Martin and Orkin, 1990) and two zinc fingers: the C-terminal finger binds DNA and the N-finger stabilises DNA binding at GATA sites (Trainor et al., 1996). In addition to binding DNA the N-finger can also interact with several co-factors, including the recently identified Friend of GATA-1(FOG-1) (Tsang et al., 1997), which can both co-operatively activate (Tsang et al., 1997) and also inhibit (Fox et al., 1999) GATA-1-mediated activation. GATA-1 is regulated by the transcriptional co-activator CBP (Blobel et al., 1998). Since CBP can associate with molecules which possess histone acetylase activity and complexes containing RNA pol II this interaction (1) suggests GATA-1 regulation through recruitment of a histone acteyltransferase complex and (2) provides a link between GATA-1 and components of the basal promoter machinery. It

must be noted that GATA-1 expression is not restricted to erythroid cells, indeed its detection in megakaryocytes, mast cells, multipotent progenitors and early monocytic cells suggests that it plays a role in the differentiation of multiple blood lineages (reviewed in Orkin, 1998).

The *SCL/TAL1* gene, a member of the bHLH family, can bind E box DNA sequences by forming heterodimers with bHLH proteins encoded by the E2A, HEB or E2-2 genes (Voronova and Lee, 1994 and Hsu et al., 1991). Gene targeting experiments in mice reveal that this gene is essential for yolk-sac erythropoiesis and *SCL* null embryonic stem (ES) cells fail to contribute to any haematopoietic lineages in chimaeric mice (Orkin and Zon, 1997)—indicating the role of *SCL* is cell autonomous. SCL, similar to GATA-1, is normally expressed in erythroid cells, mast cells, megakaryocytes and progenitor/ "stem cells" (Green et al., 1991 and Visvader et al., 1991). Constitutive expression of wild-type and dominant negative forms of SCL in erythroleukemia cell lines provide indirect support for a role of *SCL* in erythropoiesis (Aplan et al., 1992). Several studies have also suggested a role for *SCL* in vascular differentiation. Forced expression of SCL in zebrafish embryos resulted in an expansion of haematopoietic and vascular precursors (Gering et al., 1998). Moreover in the zebrafish mutant *cloche*, expression of SCL cDNA partially rescues defects in haematopoietic and vascular lineages, implying that *SCL* lies downstream of *cloche* in the developmental cascade (Liao et al., 1998).

Other critical genes involved in erythropoiesis include erythroid krüppel-like factor (*EKLF*) and the LIM domain protein, *LMO2*. Null mutations in either of these genes,

GATA-1 or *SCL* result in a similar mutant phenotype—a complete lack of erythropoiesis in the yolk sac—which implicated some regulatory interplay or common role for these genes in haematopoietic development. (Fujiwara, 1996 Warren et al., 1994, Shivadasani et al., 1995 and Robb et al., 1995). Several studies have identified various associations between these proteins. Indeed, a large multiprotein complex was discovered by *in vitro* analyses of a protein DNA complex containing GATA-1, LMO2, SCL/TAL1, E2A (a basic HLH protein) and LBD1 (another LIM-domain protein) that binds a DNA sequence consisting of E box and GATA sites separated by approximately 9bp (Wadman et al., 1997). Furthermore, since GATA-1, NF-E2 and EKLF all directly interact with and can be regulated by CBP (reviewed in Blobel et al., 2000) it is tempting to speculate that all three proteins co-operate to form a high molecular weight complex linked through CBP at the locus control region (LCR) of the β-globin gene cluster. Indeed it would appear from the increasing number of factors known to directly bind GATA-1 that the presence of GATA-1 binding sites may be central in recruiting factors to multiprotein complexes which activate distinct target genes during erythropoiesis.

2.2.1.2Transcriptional Control of Myelopoiesis

PU.1, the product of the *PU.1/SPI-1* proto-oncogene, is a member of the ETS transcription family (Klemsz et al., 1990). All ETS factors are characterised by a conserved DNA binding domain of 80 amino acids (Karim et al., 1990). PU.1 is preferentially expressed in myeloid and B cells and plays a pivotal role in their development (Chen et al., 1995A). Indeed, *PU.1* knockout mice lack neutrophils, monocytes and B cells (Scott et al., 1994) and others have shown PU.1 expression to

instruct multipotent progenitors to differentiate along the myeloid lineage (Nerlov and Graf, 1998). The structure of PU.1 is that of a winged HTH DNA binding domain (Kodandapani et al., 1996) at the C-terminus and an activation domain at the N-terminus that is believed to interact with other regulatory proteins (Klemsz et al., 1996). PU.1 regulates the expression of almost all characterised myeloid genes, including growth factor receptors. In particular, it directs macrophage specific expression of the M-CSF receptor (Zhang et al., 1994 and Olson et al., 1995), which is critical for monocytic cell survival and proliferation. Interestingly, a recent study has shown that PU.1 can directly interact with GATA-1 and as such GATA-1 negatively regulates myeloid differentiation (Nerlov et al., 2000). On the other hand, overexpression of PU.1 can block erythroid differentiation (Rektman, 1999) by interacting with and repressing GATA-1-mediated transactivation. Thus, it is postulated that the opposing effects of these factors may determine lineage-specific differentiation. These studies indicate that *PU.1* is both a "master" regulator of myeloid differentiation and a regulator of erythroid development.

AML-1 is a member of the core binding factor (CBF) or polyoma enhancer binding protein 2 (PEBP2) family of transcription factors. CBF factors consist of heterodimers between DNA binding α subunits and a β subunit that is unable to bind DNA but enhances the binding of the α subunit (Wang et al., 1993). Several α subunits have been detected including CBFA1, CBFA2 and CBFA3 (Miyoshi, 1995). The CBFα contains the runt domain which shares homology with the *Drosophila* pair-rule segmentation gene, *runt*. The runt homology domain allows binding of AML1 to its target sequence TGYGGTY and binding to CBFβ which increases DNA binding affinity (Ogawa, 1993).

AML1 knockout studies demonstrated the importance of this gene in development. The *AML1* knockout mice have a severe disruption in definitive haematopoiesis with defects in all haematopoietic lineages (Okuda et al., 1996). Recent studies have demonstrated that *AML1* can act as a repressor and an activator or transcription in a cell-specific manner. For example AML1 interacts directly with the bzip domain of C/EBP to synergistically activate the M-CSF receptor promoter (Zhang et al., 1994). More recently, AML1 was found to co-operate with LEF-1 to repress transcription of the T-cell receptor α enhancer (Levanon et al., 1998). It is speculated that *AML1* is a "master" regulator that facilitates the assembly of protein complexes for transcriptional regulation during the differentiation of primitive cells into definitive haematopoietic elements.

C-MYB, the cellular homologue of the v-myb oncoprotein, is a sequence-specific transcriptional activator (Biedenkapp et al., 1988). C-MYB is highly expressed in immature haematopoietic cells and is down-regulated during terminal differentiation of these cells (Westin et al., 1982 and Gonda and Metcalf, 1984). Moreover, chemically or cytokine-induced differentiation of myeloid or erythroid leukaemia cells results in a down-regulation of *C-MYB* (Westin et al., 1982, Gonda and Metcalf, 1984 and Kuehl et al., 1988). Furthermore, forced expression of *C-MYB* inhibits erythroid differentiation (Clarke et al., 1988), whereas antisense oligonucleotide inhibition is accompanied by growth arrest and reduced haematopoietic colony formation (Gewirtz and Calabretta, 1988). These results suggest that down-regulation of *C-MYB* is necessary for terminal differentiation. Consistent with this, *c-myb* knockout mice are severely anameic and die

in utero at the foetal liver stage (Mucenski et al, 1991). Interestingly, primitive erythropoiesis in the yolk sac and megakaryopoiesis in the liver are unaffected in these embryos. Therefore, it is believed that either *C-MYB* is required for the proliferation of multipotential progenitors or that it is required independently for the proliferation of multiple haematopoietic lineages.

Several studies have provided evidence that the retinoic acid receptor (RAR) may be key for myeloid differentiation. For example vitamin A deficiency is associated with defective haematopoiesis and retinoids stimulate granulopoiesis (Wolbach et al., 1925, Hodges et al., 1977 cited in Tenen et al., 1997). Furthermore retinoic acid (ATRA) can induce myeloid differentiation in HL60 cells and in primary leukaemic cells (Collins et al., 1990). The RARα protein is divided into six conserved domains. The C-terminal domain contains to C_2C_2 zinc finger motifs. Through this domain RARα binds to retinoic acid response elements (RAREs). RAREs consist of a direct repeat (A/G)G(G/T)TCA separated by 2 or 5 nucleotides. RARα binds as a heterodimer to this site along with the retinoic acid receptor X (RXR) which stimulates transcription in response to ATRA (Umesono et al., 1991). The importance of *RAR*α in myeloid differentiation was recognised when Collins et al. (Collins et al.,1990 and Damn et al., 1993) developed an HL60 cell which could not differentiate by ATRA treatment. RARα was mutated in this cell line but differentiation of these cells by ATRA was restored upon infection with a retrovirus expressing the wildtype receptor. Moreover, RXRα overexpression in the resistant cells restored myeloid differentiation, which suggested that the mutant receptor acted as a dominant negative by heterodimerising with wildtype RXR forming an inactive

complex. The overexpressed RXR is believed to recruit other RAR isoforms in the cell to overcome the block and activate the differentiation program. Similarly it is believed that these isoforms which may compensate for RARα in RARα knockout mice show no major defects in haematopoiesis.

A number of others transcription factors have been implication in the regulation of normal myeloid development including the myeloid zinc finger protein *MZF-1*, the Wilms Tumour Suppressor Gene (*WT-1*), ETS factors (including *SPI-B*, *FLI-1*, *ELF-1*, *ETS-2*), various *HOX* genes, Mixed Lineage Leukaemia protein (*MLL*), *C-MYC, p53*, *C-JUN* and *NF-κB* (reviewed in Tenen et al., 1997). However the proposed intricacies of their function within myelopoiesis will not be discussed here.

Interestingly, the most frequent targets of chromosomal translocations in the acute leukaemias are genes that encode these haematopoietic transcription factors, which merits the importance of these "master" regulators in the control of blood cell development. Considerable progress within this field can be attributed to a combination of genetic analyses in mice deficient in haematopoiesis-specific nuclear regulators, the molecular cloning of translocation breakpoints and mechanistic analyses of these factors in appropriate in vitro model systems (Shivadansi and Orkin, 1996, Rabbitts, 1994, Rowley, 1998, Rowley, 1999 and Sanchez-Garcia, 1997). Subsequent studies uncovered (1) how some of these proteins interact with each other to regulate transcription (2) how interactions with DNA can modulate chromatin configuration and (3) the role of some of these factors in leukaemogenesis.

1.3 Nuclear Oncogenes in Leukaemogenesis

Normal haematopoiesis is a developmental continuum characterised by an intrinsic flexibility that ensures both the maintenance of circulating blood cells and a prompt adaptation to abnormal circumstances (for example, blood loss and infection) that alter tissue homeostasis. This process requires highly orchestrated processes for its maintenance, suggesting that even the slightest imbalance could have immediate, dramatic consequences. Leukaemia is envisioned as an extreme manifestation of an uncoupling of proliferation and differentiation within the haematopoietic pool, which perturbs the relationship between resting, cycling and terminally differentiated cells. Over the last quarter of a century, many experimental data have collectively implicated loci involved in the regulation of cell proliferation as being the genomic defects of cancer cells. On the basis of their biochemical activity, where known, and the type of genetic alteration that affects the transformed cell, "cancer genes" have been divided into two broad groups: proto-oncogenes and tumour suppressor genes. The proto-oncogenes code for proteins that are components of the cellular signalling pathways such as transcription factors, growth factors and cell cycle components. Proto-oncogene alterations (such as point mutants, gene amplification or translocations) lead to a subsequent gain-of-function that accelerates cell division. These changes are dominant and normally affect only a single allele of the gene. Tumour suppressor genes, on the other hand, are subject to loss of function recessive alterations most commonly through deletions or point mutations that truncate the protein or alter an essential functional domain. It is widely accepted that tumourigenesis involves a multi-step process involving several intrinsic and extrinsic

genetic changes all acting synergistically to maintain the transformed phenotype of the "diseased" cells.

1.3.1 Activation of Proto-oncogenes

1.3.1.1 Chromosomal Translocations and Insertions

As early as 1900 scientists proposed that chromosomal abnormalities were directly related to the development of neoplasia. However, it was not until after the discovery of chromosomal banding techniques in the late 1960s that consistent recurring gains and losses of chromosomes in leukaemias, lymphomas and some solid tumours were identified (Rowley et al., 1975 cited in Rowley, 1998) (See Table 1). Moreover, the discovery of non-random, somatically acquired chromosomal translocations and inversions led to a remarkable surge of knowledge regarding the genetic changes in leukaemic cells. Over 150 different tumour-specific breakpoints have now been recognised with a remarkable 65% and 100% of acute leukaemias and chronic myeloid leukaemias harbouring chromosomal rearrangements. The most frequent targets of these chromosomal translocations or inversions turned out to be genes that encode transcription factors involved in normal haematopoiesis.

Many of these transcription factors are converted into activated oncoproteins in certain neoplasias after translocation by two principle mechanisms (Figure 4). (1) Recombinase error results in the activation of normally silent proto-oncogenes. (2) More commonly,

breaks occurring within introns between coding regions of two transcription factors genes in separate chromosomes, create a fusion gene encoding a chimaeric protein with unique properties.

1.3.1.1.1 Translocation-Generated Oncogenes in Myeloid Leukaemias

The translocations involved in the inappropriate activation of transcription factors in myeloid leukaemias show remarkable specificity for haematopoietic cells blocked in defined stages of differentiation (reviewed in Shivadansi and Orkin, 1996, Rabbitts, 1994, Rowley, 1998, Rowley, 1999 and Sanchez-Garcia, 1997). These observations implied that the resultant oncoproteins interfere with specific transcriptional networks that normally function concurrently with growth factors and their receptors to regulate haematopoiesis. Indeed, this property has been reinforced by gene targeting experiments whereby null mutations are introduced into mutant mice—specific examples of which are discussed below.

Acute Myeloid Leukaemia (AML)

AML is a clonal, malignant disease of the haematopoietic tissue. It is characterised by the proliferation of leukaemic blast cells primarily in the marrow and impaired production of normal blood cells. The leukaemic cell infiltration is accompanied nearly invariably by anaemia and thrombocytopenia. AML is classified on the basis of the morphological appearance of the bone marrow into seven subtypes, FAB M1-M7 which differ depending on the predominant cell type involved.

Acute Myeloid Leukaemia-1 (AML-1) fusions

Translocations that disrupt the *AML1* gene are amongst the most common aberrations found in human leukaemias. The t(8;21) found in 15% of myeloid leukaemias gives rise to the AML1-ETO transcriptional repressor (Erickson et al., 1992 Feinstein et al., 1995, Miyoshi et al., 1991 and Nisson et al., 1992). The t(3;21) results in the production of several fusion proteins namely AML1/EAP, AML1/MDS-1 and AML-EVI-1 (Mitani et al., 1994 and Nucifora and Rowley, 1995). inv(16) fuses the AML1 DNA binding partner, CBFβ to a smooth muscle myosin heavy chain gene, MHY11 giving CBFβ-MYH11 in 15% of acute myeloid leukaemias (Liu et al., 1993). Studies carried out with these fusions, (Klampfer et al., 1996, Mitani et al., 1994 and Liu et al., 199, respectively), suggest that they may act as dominant negatives to mediate oncogenic changes, either by producing a transcriptionally inactive dimer or by forming a complex that actively interferes with other components of the transcriptional apparatus.

Acute Promyelocytic Leukaemia (APL)

Acute promyelocytic leukaemia (APL) accounts for approximately 15% of all AMLs and comprises the M3 subtype (Chen et al., 1996 cited in Strout et al., 1997). The APL disease is characterised by the accumulation of leukaemic promyelocytes in the bone marrow, and unique to other AML forms, is frequently sensitive to all-*trans* retinoic acid (ATRA).

Retinoic Acid Receptor α (RARα) fusions

APL is currently associated with four different gene rearrangements fusing RARα to the promyelocytic leukaemia gene (PML), promyleocytic leukaemia zinc finger (PLZF), nucleophosmin (NPM), or nuclear matrix associated (NuMA) genes, which lead to the formation of reciprocal fusion proteins (N-RARα and RARα-N). RARα-PML is created by t(15:17) (Goddard et al., 1991), RARα-PLZF by t(11;17)(q23;q21) (Licht et al., 1995), RARα-NMA by t(5;17)(q32:q21) (Brunel et al., 1995) and RARα-NuMA t(11;17)(q13;q21) (Wells et al., 1996). The multimerisation of N-RARα fusion proteins are believed to interfere with normal ATRA-mediating signalling and sequestering cofactors. In particular, the involvement of PML- and PLZF- RARα fusions in APL pathogenesis have gained much notoriety in the past few years as a important paradigm for differentiation therapy (Redner et al., 1999 and Melnick and Licht, 1999 and references therin). Both PML-RARα and PLZF-RARα inhibit the activity of wildtype RARα in a dominant negative fashion (Chen et al., 1996B and Licht et al., 1996, respectively). Most commonly, APL is associated with the expression of PML-RARα and is sensitive to pharmacological levels of ATRA treatment which release repressive nuclear corepressor complexes and induce myeloid differentiation (reviewed in Fenaux et al., 1996). However, APL patients which harbour the PLZF-RARα fusion are insensitive to ATRA treatment and have an overall poor prognosis (Chen et al., 1996B). This is the result of a ligand insensitive association of a corepressor with the PLZF protein (David et al., 1997, Lin et al.,1998 and Grignani et al., 1998).

Chronic Myeloid Leukaemia (CML)

Chronic myelocytic leukaemia is a clonal disorder of the pluripotent haematopoietic stem cells which usually has a biphasic course. The initial phase is characterised by clonal expansion of a CML clone with terminal differentiation, which is followed by an acute blastic crisis phase which results in cell proliferation, maturation arrest and karyotypic clonal evolution.

The BCR-ABL Fusion

The reciprocal translocation t(9;21)(q34;q11), a hallmark of the chronic phase of CML (De Klein et al., 1982), generates the BCR-ABL fusion on the Philadelphia chromosome, which is now detected in almost 100% of patients with clinically typical CML (reviewed in Osarogiagbon et al., 1999). The product of the BCR-ABL chimera is an abnormal tyrosine kinase, $p210^{bcr\text{-}abl}$, which gives haematopoietic cells the characteristics of excessive proliferation (Daley et al., 1990), resistance to physiologic apoptotic signals (McGahon et al., 1994) and resistance to chemotherapy (Chomel et al., 2000). Interestingly, the BCR-ABL fusion has also been identified in AMLs (Najfeld et al., 1998) suggesting that it arises in primitive cells with multilineage potential.

In summary, most of these oncogenes activated/created by chromosomal abnormalities are likely to play a role in tumour-specific cell differentiation and/or proliferation controls due to their homology or identity to genes involved in other experimental systems that affect development or differentiation. Subversions in these orderly processes result in

blockades allowing the accumulation of immature proliferating cells or tumour cells. Moreover, it is hypothesised that the formation of aberrant multi-protein complexes, which occur after chromosomal translocations is paramount to tumour development.

Some other examples of proto-oncogene activation include gene amplification and retroviral insertions:-

1.3.1.2 Gene Amplification

Many cancer cells contain multiple copies of structurally normal oncogenes. These amplifications are generally associated with more advanced tumours (reviewed in Schwab, 1998).

1.3.1.3 Retroviral Insertions

Common sites of integration for animal tumour viruses are near or within proto-oncogenes causing their activation: cellular genes are brought under control of the viral LTR or are fused to viral sequences with the consequent production of novel proteins (Hayward et al., 1981). This knowledge has led to the use of insertional mutagenesis as a means of identifying novel proto-oncogenes that are consistent targets of proviral insertion (Nusse and Varmus, 1982).

One specific nuclear oncogene identified in this way was Ecotropic viral integration site-1 or *EVI-1* (Morishita et al., 1988). Subsequent work has shown that *EVI-1* encodes a zinc finger protein, is essential for foetal and adult development and that unscheduled *EVI-1* expression is associated with myeloid leukaemias and myelodysplastic syndromes in mice and humans. The remainder of this chapter discusses the properties of the *EVI-1* transcription factor in detail.

1.4. The Evi-1 Transcription Factor

1.4.1 Identification of Evi-1

EVI-1 was discovered using AKXD recombinant inbred (RI) mouse strains derived from crosses of AKR/J and DBA/2J. The high levels of leukaemia susceptibility in AKXD strains are the result of inheritance of endogenous ecotropic MuLV proviruses, namely Emv-11 and Emv-14 (Mucenski et al., 1986, Gilbert et al., 1993). Tumourigenesis involves the expression of these endogenous ecotropic proviruses, recombination between somatic viruses, and re-integration near cellular oncogenes. The DNA from all tumours of the strain AKXD 23 was found to contain proviruses that were inserted into the same chromosomal domain designated ecotropic viral integration site 1 (*evi-1*) (Mucenski et al., 1988). In a separate study *evi-1* activation by retroviral insertions was found in IL-3-dependent myeloid leukaemia cell lines (Mucenski et al., 1988). In light of this knowledge further studies detected *evi-1* rearrangements in myeloid cell lines and

leukaemias in NSF/N or NSF/N hybrid mice injected as neonates with wild mouse ecotropic or MCF-MuLV and Cas-Br-E MuLV (Mucenski et al., 1988, Bergeron et al., 1992). *Evi-1* insertions are predominantly myeloid-specific. However, some have been found is a small number of B and pre-B cell tumours (Mucenski et al., 1988). The subsequent elucidation of the intron-exon structure of the 5' end of the gene revealed that proviral insertions were oriented in the same transcriptional direction (5'→3'), and were tightly clustered, occurring within a 0.6Kb stretch of DNA. The intron-exon structure of *EVI-1* is not characterised. However, the genomic organisation of the first three exons is known and translation is initiated in exon three (Matsugi et al., 1990). In humans, *MDS1* was recently identified as a previously unreported exon of *EVI-1* with the addition of this exon creating the *MDS1/EVI-1* isoform. *MDS1* has been mapped 170-400Kb upstream of *EVI-1* (Nucifora et al., 1994). (*MDS-1* is discussed later in sections 1.4.3.1 and 1.4.6.1).

A second common integration site associated with evi-1 has also been identified in Friend-virus induced murine leukaemias. Nearly a quarter of all myeloblastic leukaemias induced by this virus were found to exhibit proviral insertions in this particular site which was then named fim-3 (Borderaux et al., 1987). Characterisation of another common integration site, cb-1, demonstrated that it was identical to fim-3 (Bartholomew et al., 1989). It was subsequently shown that these proviral insertions also activate evi-1 expression. Genomic organisation of the cb-1/fim-3/evi-1 region shows that fim-3 is located 90Kb upstream of the first exon of evi-1(Bartholomew and Ihle, 1991). It is believed that these insertions act from a distance to activate evi-1 transcription by an enhancer-insertion mechanism. Ectopic expression of evi-1 in murine myeloid

leukaemias is therefore a consequence of retroviral insertion at either the fim3 or evi-1 loci.

1.4.2 Development and Evi-1

To determine the molecular mechanisms of *EVI-1* action in leukaemogenesis it is imperative that we understand the normal function and regulation of the *EVI-1* gene.

1.4.2.1 Evi-1 and mds1/evi-1 expression in adult murine tissues

EVI-1

The zinc finger regions of *EVI-1* share homology with egl-43, a zinc protein in *C.elegans* which is involved in migratory activity and development (Garriga et al., 1993). Primary northern blot analyses of murine tissues have revealed high *evi-1* levels in the kidney, uterus, lung and heart (Morishita et al., 1990 and Perkins et al., 1991B, Wimmer et al., 1998). Therefore, the restricted spatiotemporal pattern of *evi-1* expression in adult tissues suggests that evi-1 is involved in organogenesis.

During examination of cell lines, *EVI-1* has not been detected in myeloid, T-cell and B-cell leukaemic cell lines and cell lines derived from a range of organs, including lung, placenta, ovary, spleen and heart in these studies (Morishita et al., 1990). *EVI-1* is

expressed in primary foetal kidney cells but not in many established kidney cell lines (Bartholomew and Clark, 1994).

MDS1/EVI-1

The evolutionarily conserved *MDS1* gene shares homology with the PR domain of the retinoblastoma binding zinc finger protein, RIZ (Buyse et al., 1995 and Fears, 1996), to a lesser extent the PR domain of the transcriptional repressor PRDI-BFI/Blimp 1 (Huang et al., 1994) and egl-43 (Nucifora et al., 1997). The fusion transcript between *evi-1* and *mds-1*, described in section 1.4.1 earlier, has also been detected in normal murine tissues, including haematopoietic progenitor cells, pancreas, kidney and lung (Sigurdsson et al., 1995 and Wimmer et al., 1998). Wimmer et al., 1998 demonstrated that endogenous levels of evi-1 and mds-1/evi-1 when up-regulated in mouse embryonic stem cells induced them to differentiate into myeloid haematopoietic cells *in vitro.* Indeed, *mds1/evi-1* expression overlaps remarkably with *evi-1* expression patterns in murine tissues, with no major shift in their relative abundance (Wimmer et al., 1998). Furthermore, considering *MDS1/EVI-1* expression is down-regulated in leukaemic samples relative to overexpressed *EVI-1* levels and the transcriptionally antagonistic nature of these proteins (Solderholm, 1997), finely tuned levels of these isoforms may be of great importance for either normal or leukaemic development.

1.4.2.2 Evi-1 Expression in Murine Embryonic Development

Whole mount *in situ* hybridisation studies of murine embryos revealed that on days 9.5, 12.5 and 14.5 post-coitum high level evi-1 expression was evident in the urinary system, the Müllerian ducts, the limb buds, endothelial cells and valve leaflets of the heart, the tracheal bronchial epithelium of the lungs and epithelial cells of the developing nasal cavity (Perkins et al., 1991). Interestingly, this restricted pattern of gene expression overlaps with that of other transcriptional regulatory genes (including some *HOX* and *RAR* genes) (Perkins et al., 1991). Thus, it has been postulated that zinc finger proteins such as *EVI-1* may function in murine development by controlling other important regulatory genes such as above. Indeed in *Drosophila*, zinc finger proteins are heavily implicated in cell fate and decisions concerning body plan (Chavrier et al., 1990).

Evi-1 knockout experiments were subsequently performed to address its' biological function in murine development (Hoyt et al., 1997). Homozygous *evi-1* knockout mice lack the full length *evi-1* transcript and die in utero at 10.5 days post-coitum (d.p.c.). In comparison to normal littermates at 10.5 d.p.c. multiple malformations are apparent: The most obvious being an reduced foetal size, a malformed forebrain, large pericardial sacs and large-scale haemorrhaging. Furthermore, disruption of paraxial mesechyme ventral to the neural tube was evident along with defective myotome at somite-regions. It is noteworthy that tissues which normally express high levels of *evi-1*, including kidney and ovary, are relatively unaffected in *evi-1* homozygous mutants. However, the knockout mice used in these experiments are not null mutants as they continue to express the

continue to express the alternatively spliced *evi-1* transcripts. Therefore, these *evi-1*-expressing tissues may be somehow salvaged from more detrimental knockout effects by the compensatory effects of evi-1 isoforms.

1.4.3 EVI-1 is an Oncogene Involved in Human Disease

1.4.3.1 Myelogenous Leukaemias

Ectopic expression of *EVI-1* has been correlated with the progression of human malignancies namely, acute myelogenous leukaemia (AML), myelodysplastic syndrome (MDS) and chronic myelocytic leukaemia in blastic crisis (CML-BC).

Accumulated evidence demonstrates that *EVI-1* is transcriptionally activated in these leukaemias by translocations and inversions involving chromosome 3q26—where *EVI-1* is located—which include t(2;3)(p15;q26), inv(3)(q21q26), t(3;3)(q21;q26), t(3;7)(q27;q22), t(3;12)(q26;p13), t(3;13)(q26;p13-14), t(3;17)(q26;q22) and t(3;21)(q26;q22) (reviewed in Nucifora, 1997).

In general, 3q21q26 aberrations and *EVI-1* activation in myeloid leukaemias are correlated with abnormalities in megakaryopoiesis which is characterised by high platelet counts (Bitter et al., 1985, Bernstein et al., 1986) and lack of response to conventional chemotherapy (Testoni et al., 1999). However, it is likely an array of additional genetic

lesions contributes to leukaemogenesis associated with unscheduled *EVI-1* expression. For example, myelogenous leukaemias have been described with simultaneous 3q21q26 abnormalities and monosomy 7 (Igarashi, 1998).

Acute Myeloid Leukaemia (AML)

In AML cases with inv(3)(q21q26) and t(3;3)(q21q26), *EVI-1* is located near the q26 breakpoint, while the ubiquitously expressed *Ribophorin I* gene is located 50kb upstream near the q21 breakpoints. As a consequence of these rearrangements, *Ribophorin I* is positioned transcriptionally towards either the 3' end of the *EVI-1* gene in the case of inv (3) or towards the 5' end of the *EVI-1* gene in t(3;3). This indicates that *Ribophorin I* provides enhancer elements for *EVI-1* transactivation (Suzukawa et al., 1994).

Chronic Myeloid Leukaemia (CML)

The reciprocal translocation t(9;21)(q34;q11) is a hallmark of the chronic phase of CML (CML-BC) (De Klein et al., 1982) resulting in the generation of the $p210^{bcr\text{-}abl}$ fusion gene on the Philadelphia (Ph) chromosome (Ben-Neriah, 1986). Subsequently, a series of additional aberrations accumulate resulting in blastic crisis. t(3;21)(q26;q22) is one of these additional alterations in CML-BC (Rubin et al., 1987). This particular translocation is also seen to a lesser extent in MDS and secondary leukaemias (so called therapy-related MDS or Acute Lymphoblastic Leukaemias) (Bitter et al., 1985)—discussed below. This

raises the possibility that the molecular event underlying this reciprocal translocation has a critical role in the progression from a preleukaemic state to a leukaemic state.

EVI-1 Fusion Genes

The t(3;21)(q26;q22) can produce the chimaeric protein, AML1-EVI-1 that results from *AML1* and *EVI-1* crossing the t(3;21) breakpoint resulting in a fusion of the N-terminal runt homology domain of *AML1* to the *EVI-1* gene (Mitani et. al., 1994). Interestingly, between the t(3;21) breakpoint and *EVI-1* there are two other genes *EAP*, which encodes the ribosomal protein L22 and *MDS1*, which encodes a small polypeptide of unknown function (Nucifora et al, 1994). All three chromosome 3 genes are in the same transcriptional orientation, directed away from the breakpoint site, and in the same orientation as *AML1*. These recombinant chromosomes exhibit eight different chimaeric RNA transcripts including AML1/MDS1/EVI-1 and EAP/MDS1/EVI-1 (Fears et al., 1995, Zent et al., 1997). In addition, *TEL1(ETV6)*—recently discovered as a potent sequence specific transcriptional repressor (Lopez et al, 1999)—is one of the most frequently rearranged transcription factors in human leukaemias and has been documented as a fusion protein with MDS1/EVI-1 as a result of t(3;12)(q26;p13) (Peeters et al., 1997). Generally, it is hypothesised that these fusion proteins act dominantly over the endogenous proteins necessary for normal haematopoiesis.

Myelogenous leukaemia expressing EVI-1 without 3q26 abnormalities

The myelodysplastic syndromes (MDSs) are a subset of haematological malignancies with altered proliferation and differentiation of the erythroid and myeloid lineages of which approximately 30% of cases will progress into full blown AML. MDSs are characterised into 3 categories; refractory anaemia (RA) is followed by increasing advanced disease states refractory anaemia with ringed sideroblasts (RARS), RA with excess of blasts (RARE) and RA with excess of blasts in transformation (RAEBT).

Some studies have reported the incidence of *EVI-1's* expression in MDS patients as generally low with ectopic *EVI-1* expression detected without chromosome 3q26 abnormalities in primarily RAEB and RAEBT (Russell et al., 1994 and Fichelson et al., 1992, Dreyfus et al., 1995). As with CML-BC it appears that *EVI-1s* appearance is associated with leukaemia progression.

Chromosomal abnormalities involving 3q26 have been established in less than 5% of human leukaemias. However, recent findings strongly argue that overexpression of *EVI-1* in MDSs and AMLs without 3q26 abnormalities is much more common and suggests a major role in tumourigenesis for these neoplasias (Ogawa et al., 1996). Expression of *EVI-1* without 3q26 aberrations has now been documented in 70% of CML-BCs, 20.3% of AMLs and 27.3% of MDS-derived leukaemia (Ogawa et al., 1996). Others have also detected abnormal *EVI-1* expression in acute promyelocytic leukemia (APL) without 3q26 structural abnormalities (Russel et al., 1994).

However, these results must be interpreted with caution: a number of possibilities exist which may explain the prevailing *EVI-1* expression associated with these malignancies; (1) it may simply be a consequence of a small deletion: this is exemplified by the *SCL* oncogene, which was originally cloned from the breakpoint of t(1;14)(p32;q11) found in 1-3% of human T cell acute lymphoblastic leukaemia (T-ALL) (Begley et al., 1989). A functionally equivalent abnormality, not observed cytogenetically, is a small site-specific interstitial 90kb deletion event on chromosome 1 that brings *SCL* under control of *SIL* (SCL interrupting locus)—a ubiquitously expressed T cell gene. The recorded frequency of the SIL-SCL deletion event in T-ALL is 6-26% (reviewed in Begley and Green, 1999 and references therein). ; (2) activation of *EVI-1*-regulating proteins or (3) the cells may be arrested at a stage of development where *EVI-1* is normally expressed in haematopoietic development.

1.4.3.2 *EVI-1* Expression in Non-Haematological Malignancies

Using RT-PCR techniques and RNAase protection assays, high level *EVI-1* expression has been detected in ovarian cancers, endometrial carcinomas and melanomas (Brooks et al., 1996). Therefore, it is possible that *EVI-1* is also important in the progression of some solid tumours. Similar to many haematological disorders some ovarian carcinoma cell lines displayed 3q26 abnormalities, suggesting this is important in both haematological and non-haematological disorders where *EVI-1* is aberrantly expressed. As yet, the role of *EVI-1* in non-haematological tissues is unknown.

1.4.4 Structure of EVI-1

Characterisation of a 5.1Kb *EVI-1* cDNA revealed an open reading frame of 3126 nucleotides encoding a predicted 145KD C_2H_2 zinc finger protein (Morishita et al., 1988) (Figure 3). The N-terminal domain of EVI-1 comprises seven repeats of the zinc finger motif (ZF1) and there are three additional zinc finger motifs toward the C-terminus (ZF2). There is a proline-rich repressor domain (Bartholomew et al., 1997) and an acidic domain at the C-terminus of unknown function. EVI-1 is localised in the nucleus, exhibiting a dotted pattern of localisation in leukemia cells that contain the provirally active allele (Matsugi et al., 1990). The gene is conserved between humans and mice with human and mouse open reading frames 91% homologous at the DNA level and 94% homologous at the amino acid level (Morishita et al., 1990).

1.4.4.1 Alternative EVI-1 Splice Forms (Figure 3)

A shorter isoform of EVI-1, with a predicted molecular weight of 88Kd, is produced via alternative splicing in both human (Morishita et al., 1990) and mouse (Bordereaux et al., 1990). This form lacks zinc fingers six and seven and the IR domain and was designated Δ324 (since it lacked 324 amino acids altogether). In addition, an extra nine amino acids are detected within the repressor domain of both the human (Morishita et al., 1990) and mouse (Bartholomew, unpublished) Δ324. Furthermore, Δ324 isoform lacks the biological activity of the Full length EVI-1 protein (FLEvi-1) (Kilbey and Bartholomew,

1998). Another EVI-1 variant designated, Δ105 lacks 105 C-terminal amino acids (Bartholomew, personal communication) and represents 50% of EVI-1 protein in murine cells examined. This isoform retains the biological activity of the FLEvi-1 protein, indicating the truncated region is dispensable for EVI-1 function. The MDS1/EVI-1 splice form has a distinct transcription initiation site than the other EVI-1 isoforms and has an additional 188 novel amino acid residues derived form an upstream exon—designated MDS1(which contains a conserved PR domain)—at the 5' end of the previously reported EVI-1 protein (Fears et al., 1996). The functional significance of these isoforms are under investigation. (MDS1/EVI-1is discussed in sections 1.4.2.1 and 1.4.6.5).

1.4.5 Biochemical Properties of EVI-1

The discovery of ten zinc finger motifs in EVI-1 strongly suggested that it encoded a sequence-specific DNA binding protein that plays a role in transcriptional regulation. Consensus binding sites have been determined for both ZF1 and ZF2: ZF1 binds to TGACAAGATAA, which shows an overlap for the erythroid-specific GATA family of transcription factors that bind the consensus sequence motif (A/T)GATA(A/G) (Perkins and Kim, 1991 and Delwel et al., 1993) and ZF2 binds to GAAGATGAG (Funabiki et al., 1994). *In vitro* studies have revealed that EVI-1 can bind to the ZF2 site. However, only a truncated EVI-1 protein can bind to the ZF1 site (Bartholomew, 1997) which suggests that some region in EVI-1 suppresses binding of ZF1 domain to its recognition

sequence *in vitro*, raising the possibility that *in vivo* binding of ZF1 to its site may be regulated.

1.4.6 Biological Effects of EVI-1

1.4.6.1 EVI-1 functions as a Transcriptional Repressor

The EVI-1 protein acts as a repressor of gene transcription in several cell types (Bartholomew et al., 1997 and Funabaki et al., 1994) and the repressor activity resides in the proline-rich region between amino acids 514 to 724 (Bartholomew et al., 1997) (Figure 3). An additional repressor domain is located between ZF1 and the defined repressor domain shown in Figure 3. However, this region (IR) gives only partial repression (Bartholomew, 1997).

EVI-1 can suppress binding of the erythroid-specific transcription factor, GATA-1 to its recognition site (Kreider, 1993) in choloroamphenicol tranferase (CAT) reporter assays. Although no physiological GATA sites have been recognised by EVI-1, it is postulated that interactions with other transcription factors or co-ordinator proteins, may be required to increase the binding affinity. Alternatively, EVI-1 (like many of the erythroid-related transcription factors) may directly bind to GATA-1 through the GATA-1 consensus site in ZF1 to inhibit its activity.

TGFβ, one of the best-characterised cytokines, inhibits proliferation in a number of cell types (Massague et al., 1990). Indeed, the TGFβ receptor and its downstream components, are often targets for mutations in some cancers (Eppert et al., 1996 and Riggins et al., 1996). More recently, EVI-1 (Kurokawa, 1998A), was found to repress the growth-inhibitory effect TGFβ in epithelial cells by a direct interaction of the ZF1 domain with the intracellular mediator, Smad3. The Rp region is also essential for this repression, although the mechanism is unknown. Therefore the abrogation of this inhibitory factor could be one molecular mechanism whereby EVI-1 induces leukaemogenesis.

Recent studies have demonstrated that EVI-1 directly interacts with the short-range transcriptional corepressor murine C-terminal Binding Protein 2 or mCtBP2 (Turner and Crossley 1998). The recognition motif, **P-DLS-K** found in mammalian, viral and *Drosophila* proteins, mediates interactions with CtBP corepressors (Turner and Crossley et. al., 1998). Two similar motifs, **PFDLTTK** and **PLDLSNG** are located between amino acids 553-559 and 584-590 within Rp of EVI-1 (Figure 3). However, it is not known whether these sites are involved in mCtBP2 binding, or if this binding is required for repression or biological activity of the EVI-1 protein.

Little is known concerning the target genes of EVI-1 (discussed below in section 1.4.7). However, several potential EVI-1 responsive genes have been identified including

150 B9-1[1] (named after the clone number) and Inositol triphosphate type 2 receptor gene[2] (*Itrp2*), which are both down-regulated in leukaemic cells expressing EVI-1 (Matsugi et al., 1995[1] and Kim et al., 1998[2]).

1.4.6.2 EVI-1 Transforms Rat1 Fibroblasts

Rat1 cells modified to express either evi-1 or the AML1/EVI-1 fusion protein form transformed foci in soft agar assays. Interestingly, enhanced transformation is observed on transfection of p210 $^{bcr/abl}$ Rat1 fibroblasts with AML1/EVI-1 expressing constructs (Kurokawa et al., 1995). Furthermore, introducing evi-1 deletion mutants into these assays demonstrates that the DNA binding domains, ZF1[2] and ZF2[3] and the repressor domain, Rp[1] are all essential for this transforming activity (Bartholomew et al., 1997[1], Kilbey and Bartholomew, 1998[2] Kurokawa et al., 1995[3]).

The hallmark of cancer is uncontrolled cellular growth, leading to high numbers of invasive abnormal cells. In addition to altering the growth properties of several mammalian cell-types, as described above, overexpressing evi-1 also abrogates components of the cell cycle engine. In evi-1 transformed Rat1 fibroblasts the activity of the cyclin dependent kinases (CDKs) Cdk2 and Cyclin E is up-regulated, the G1 phase of the cell cycle is shortened and total cellular levels of the CDK-inhibitor p27 are down-regulated (Kilbey et al., 1999). Furthermore, evi-1 expression promotes an increase in survival potential and saturation density. Together, all of these changes are consistent with a reduced requirement for adhesion and mitogenic signals in these cells—features

common to transformed cells. Within a leukaemic setting it is envisaged increased Cdk2 activity would increase the sensitivity of haematopoietic cells to growth factors and thus impart a selective advantage on haematopoietic cells.

1.4.6.3 Evi-1 Overexpression Abrogates Haematopoietic Cell Growth

Studies have shown that ectopic evi-1 expression can block the growth of EPO responsive cells namely normal murine bone marrow progenitor cells and 32Epol cells (Kreider et al., 1993). Similarly, introduction of evi-1 or AML1/EVI-1 retroviral vectors into 32Dcl3 cells, appears to block the ability of cells to differentiate in response to Granulocyte Colony Stimulating Factor (G-CSF) (Morishita et al., 1992 and Tanaka et al., 1995, respectively). Similar studies by Khanna-Gupta et al. led to the realisation that it is level of evi-1, rather than aberrant evi-1 expression alone which is the important determinant of this differentiation block (Khanna-Gupta et al., 1996). Finally, the 3q21q26 syndrome has been closely associated with a thrombocytopenic phenotype (Bitter et al., 1985, Bernstein et al., 1986) and lack of response to conventional chemotherapy (Testoni et al., 1999). *In vitro*, overexpression of evi-1 in embryonic stem cells produces abnormally high numbers of megakaryocyte colonies—demonstrating that high levels of evi-1 are linked to abnormal megakaryopoiesis (Sitailo et al., 1999).

1.4.6.4 EVI-1 as an Activator

Studies have revealed that as well as its repressor activity, EVI-1 can also act as a transcriptional activator. EVI-1 binds to the consensus sequence GACCAGATAAGATAN$_{1-28}$CTCATCTTC in HEC1B and NIH3T3 cells using artificial constructs with ZF1 and ZF2 sequences located upstream of the minimal thymidine kinase (tk) promoter (Morishita et al., 1995). EVI-1, AML/EVI-1 and AML1/MDS1/EVI-1 also stimulate transcriptional activation of the c-fos promoter and raises AP-1 activity with ZF2-dependence (Tanaka et al., 1994, Kurokawa et al., 1995).

1.4.6.5 Biological Activity of EVI-1 Fusions—Potential Implications for Leukaemogenesis

Thus far AML1/EVI-1 [1], TEL1/EVI-1 [2], MDS1/EVI-1 [3] and AML1/MDS1/EVI-1 [4] fusion genes have been cloned (Nucifora, 1994 [1], Raynaud, 1996 [2], Sood et al., 1999 [3,4]). AML1/EVI-1 was subsequently found to dominantly suppress AML1 transactivation, leading to a block in myeloid differentiation (Tanaka, 1995). In addition, AML1/EVI-1 was shown to have a higher affinity for the AML1 binding partner, CBFβ compared to wild-type AML1(Tanaka et al., 1998)—which may account for the dominant effects of AML1/EVI-1. Indeed, AML1/EVI-1 and AML1/MDS1/EVI-1 enhance AP-1 activity, repress TGFβ signalling and transform Rat1 fibroblasts (Kurokawa et al., 1995. Kurokawa et al., 1998B), as evi-1 does (Tanaka, 1995, Kurokawa, 1998A and Kurokawa, 1995 respectively). On the other hand, mds1/evi-1 enhances TGF-β signalling and

strengthens its growth-inhibitory effect. Furthermore, whereas EVI-1 represses activation by the GATA-1 erythroid factor, MDS1/EVI-1 enhances GATA-1-mediated transactivation (Solderholm et al., 1997). In addition, whereas mds1/evi-1 has no effect on granulocytic differentiation induced by G-CSF in the haematopoietic precursor cell line 32Dcl3, the AML1/MDS1/EVI-1 fusion—similar to evi-1 alone (Khanna-Gupta, 1996)—blocks differentiation resulting in cell death. The different response of myeloid cells constitutively expressing normal or fusion evi-1 proteins to G-CSF and TGF-β may depend on the transactivation properties of these proteins resulting in divergent expression of downstream target genes regulated by these proteins. Interestingly, a recent study has demonstrated that expression of AML1/MDS1/EVI-1 in mouse bone marrow cells induces an acute myelogenous leukaemia in these animals (Cuenco et al., 2000). Whatever the exact mechanisms, it is clear that the regulation of tumour growth by chimaeric proteins will depend on the fusion partner with AML1.

Together these findings suggest that EVI-1 fusion proteins during leukaemogenesis may repress physiologically important genes or abnormally regulate genes normally repressed (or activated) by AML1 in an EVI-1-specific manner. Furthermore, direct interactions of the mSin3A corepressor with AML1 appears to be necessary for AML1-mediated repression (Lutterbach et al., 2000), suggesting that HDACs, the downstream effectors of Sin3A, are potential therapeutic targets of AMLs—in a similar manner to APL malignancies (Reviewed in Melnick and Licht, 1999).

1.4.7 Potential Targets of EVI-1

Unfortunately, our knowledge of the downstream target genes regulated by EVI-1, and indeed many oncoproteins, remains poor. Therefore, in order to bridge the gaps within contemporary models of leukaemia progression, and more specifically to ascertain a molecular role for EVI-1 in myeloid leukaemias, it is important to determine EVI-1's genetic targets within cells. Potential EVI-1 targets include the lineage-specific regulators of haematopoiesis, negative regulators of cell growth and components of the apoptotic pathway.

1.4.7.1 Subversion of Haematopoietic Lineage-Restricted Transcription Factors by EVI-1

During leukaemogenesis, *EVI-1* (and other transcriptional repressors) may inhibit the regulation of key haematopoietic modulators. As discussed at length in section 1.3, studies concerning the molecular genetics of leukaemia reveal the importance of regulatory factors in normal haematopoiesis. Since the accumulative evidence implicates ectopic *evi-1* overexpression in the disruption in erythroid (Krieder et al., 1993), myeloid (Morishita et al., 1992, Tanaka et al., 1995 and Khanna-Gupta et al., 1996) and megakaryocyte (Bitter et al., 1985, Bernstein et al., 1986, Testoni et al., 1999 and Sitailo et al., 1999) lineages it seems logical to postulate a role for EVI-1 in disrupting key regulator factors involved in the normal development of these lineages. Candidate erythroid/ megakaryocyte targets for direct or indirect *EVI-1* dysregulation during leukaemogenesis include *GATA-1*, *GATA-2*, *NF-E2* and

c-Mpl receptor, which are key regulators of erythropoiesis and megakaryopoiesis. Candidate Evi-1 targets within the myeloid lineages include the myeloid-specific regulators (section 1.2.2.1.2) such as *PU.1, HOX genes, RARs and C-MYB.*

1.4.7.2 Repression of Negative Regulators of Cell Proliferation

Another interesting possibility is that EVI-1 may down-regulate negative regulators of cell proliferation such as the cyclin-dependent kinase inhibitors (CDKI's), many of which are candidate tumour suppressor genes. As discussed in section 1.4.6.2, recent studies have shown that constitutive expression of *evi-1* in Rat1 cells down-regulates total levels of the p27 CDKI (Kilbey et al., 1999). Other possibilities include *EVI-1*-mediated repression of the p16INK4B, p12INK4A and p21 CDKI's (Hirama and Koeffler, 1995). Indeed, deletions and/or mutations of these CDKI's have been reported in a variety of human cancers/ leukaemias (Hirama and Koeffler, 1995).

1.4.8 Project Aims

The *evi-1* proto-oncogene is frequently observed in patients with certain myeloid leukaemias. In human myeloid leukaemias and murine retrovirally induced leukaemias ectopic expression of *evi-1* is a result of non-random chromosomal translocations or retroviral insertions of 3q26, respectively, where the gene is located (Bartholomew et al., 1989 and Morishita et al., 1992). More recent work has demonstrated that evi-1 is a

repressor of transcription (Bartholomew et al., 1997). Repression is mediated through a 200 base pair proline-rich repressor region—designated Rp—which is essential for the transformation of Rat1 fibroblasts by evi-1 (Bartholomew et al., 1997). However, the precise mechanisms of evi-1-mediated repression are unknown. Furthermore, no studies have addressed the relevance of the Rp domain—and other evi-1 functional domains—for the biological activity of evi-1 in haematopoietic cells. Therefore, the principle objectives of this project were to (1) utilise specific evi-1 deletions mutants as genetic tools to investigate the biological activity of evi-1 in cell lines and primary bone marrow cells, (2) to address potential mechanisms of evi-1-mediated transcriptional repression.

1.4.8.1 Investigating the functional significance of Evi-1 domains for the biological activity of Evi-1 in mammalian cells.

These studies utilised the full length evi-1 (FLevi-1) and evi-1 mutant retroviral constructs to examine the biological activity of evi-1 in an established Rat1 model system. It was postulated that several of these deletion mutants, which lack important evi-1 biological domains, could function in a dominant negative fashion and inhibit the activity of the wild-type FLevi-1 protein. Therefore, Rat1 cell lines were modified to constitutively coexpress FLevi-1 and a selection of evi-1 deletion mutants to test whether the transformed phenotype of these cell lines were reverted using the Rat1 system. Using the same retroviruses, the effects of overexpressing evi-1 in bone marrow cells were analysed. An experimental system was established whereby the production of

haematopoietic colony growth of bone marrow cells infected with retrovirus was examined in methyl cellulose colony assays. As before, mutant constructs were used in these assays to establish the importance of evi-1 functional domains for the biological activity of evi-1—but this time in a primary bone marrow experimental system.

1.4.8.2 Mechanisms of Evi-1-Mediated Biological Activity

As a transcriptional repressor, it is likely that the biological activity of evi-1 is mediated by interactions with transactivator proteins, general transcriptional machinery or cofactors such as co-repressors. Therefore, such interactions were looked for and the consequences of protein-protein interactions on the biological activity of evi-1, investigated. Mutants lacking these respective protein-binding domains were examined in *in vitro* reporter assays—established previously in our laboratory—which assay evi-1 repressor and transforming activities.

Chapter 1 Figures

Figure 1: Model of Haematopoietic Origins in the Mouse

AGM, aorta gonad mesenephros; RBC, red blood cells; LTRSC NB, long term new born-repopulating stem cells; LTRSC ADULT, long term adult-repopulating stem cells. (See section 1.2.2).

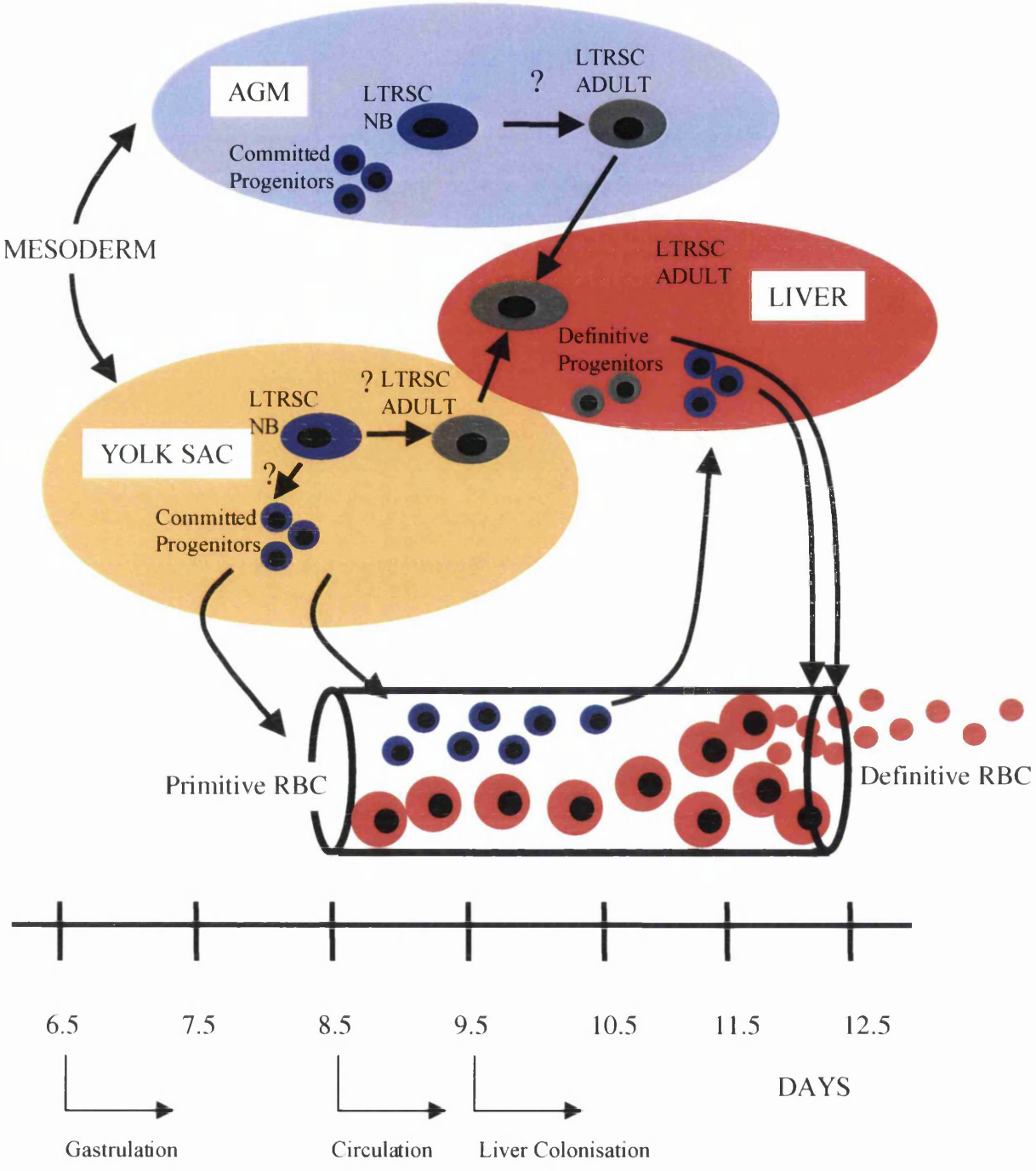

AGM
LTRSC NB
?
LTRSC ADULT
Committed Progenitors
MESODERM
LTRSC ADULT
LIVER
Definitive Progenitors
? LTRSC ADULT
LTRSC NB
YOLK SAC
?
Committed Progenitors
Primitive RBC
Definitive RBC
6.5
7.5
8.5
9.5
10.5
11.5
12.5
DAYS
Gastrulation
Circulation
Liver Colonisation

Figure 2: Cell Lineages of the Haematopoietic System: Hierarchical Model

LTRSC, long term repopulating stem cell; CFU-GEMM, colony forming unit granuolcyte/eythroid/monocyte/macrophage; CFU-GM, colony forming unit-granulocyte/macrophage; CFU-Eos, colony forming unit-Eosinophil; CFU-G, colony forming unit-granulocyte; CFU-M, colony forming unit macrophage; CFU-Baso, colony forming unit-Basophil; CFU-Meg, colony forming unit-megakaryocyte; BFU-Meg, burst-forming unit/megakaryocyte; BFU-E, burst-forming unit-erythroid; CFU-E, colony forming unit erythroid.

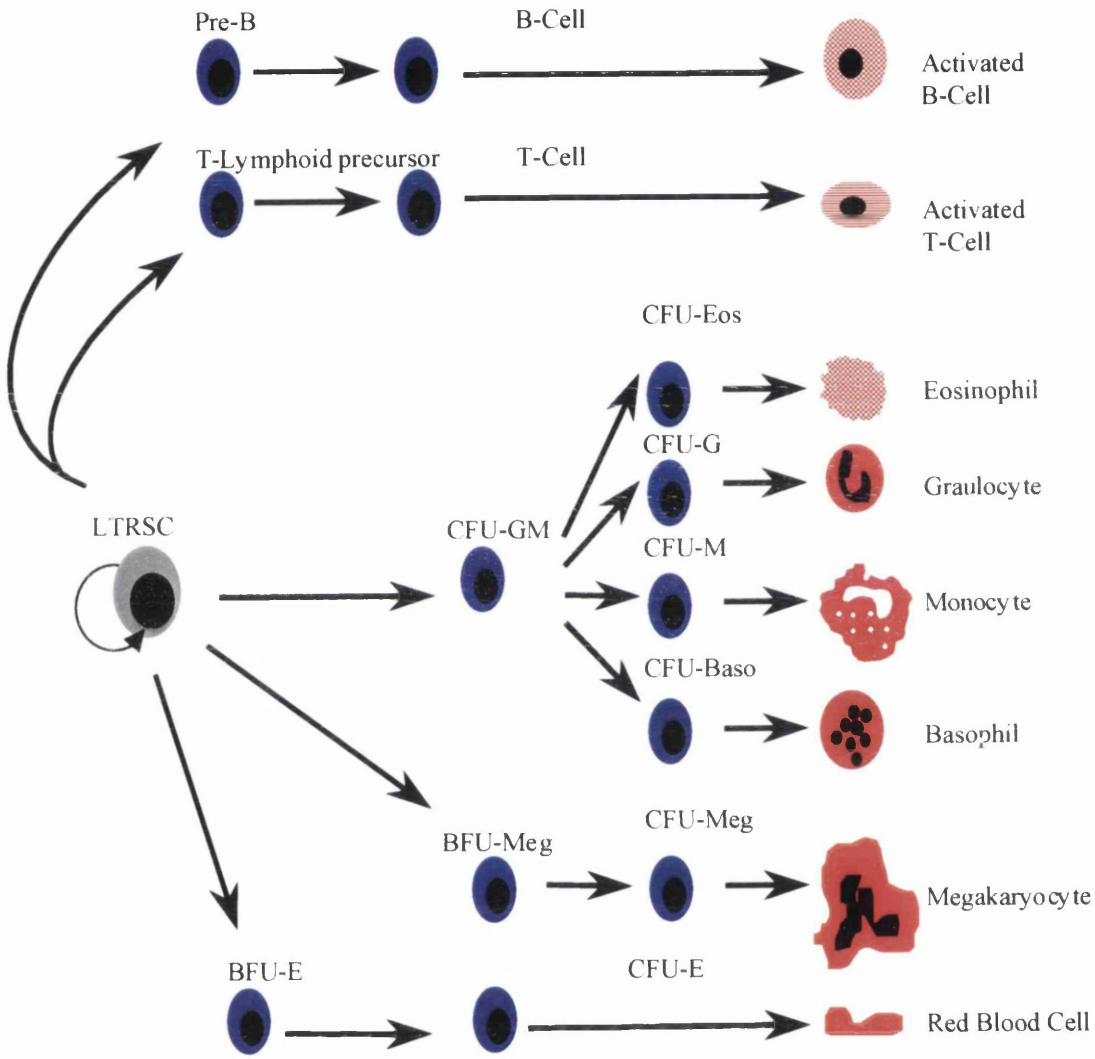
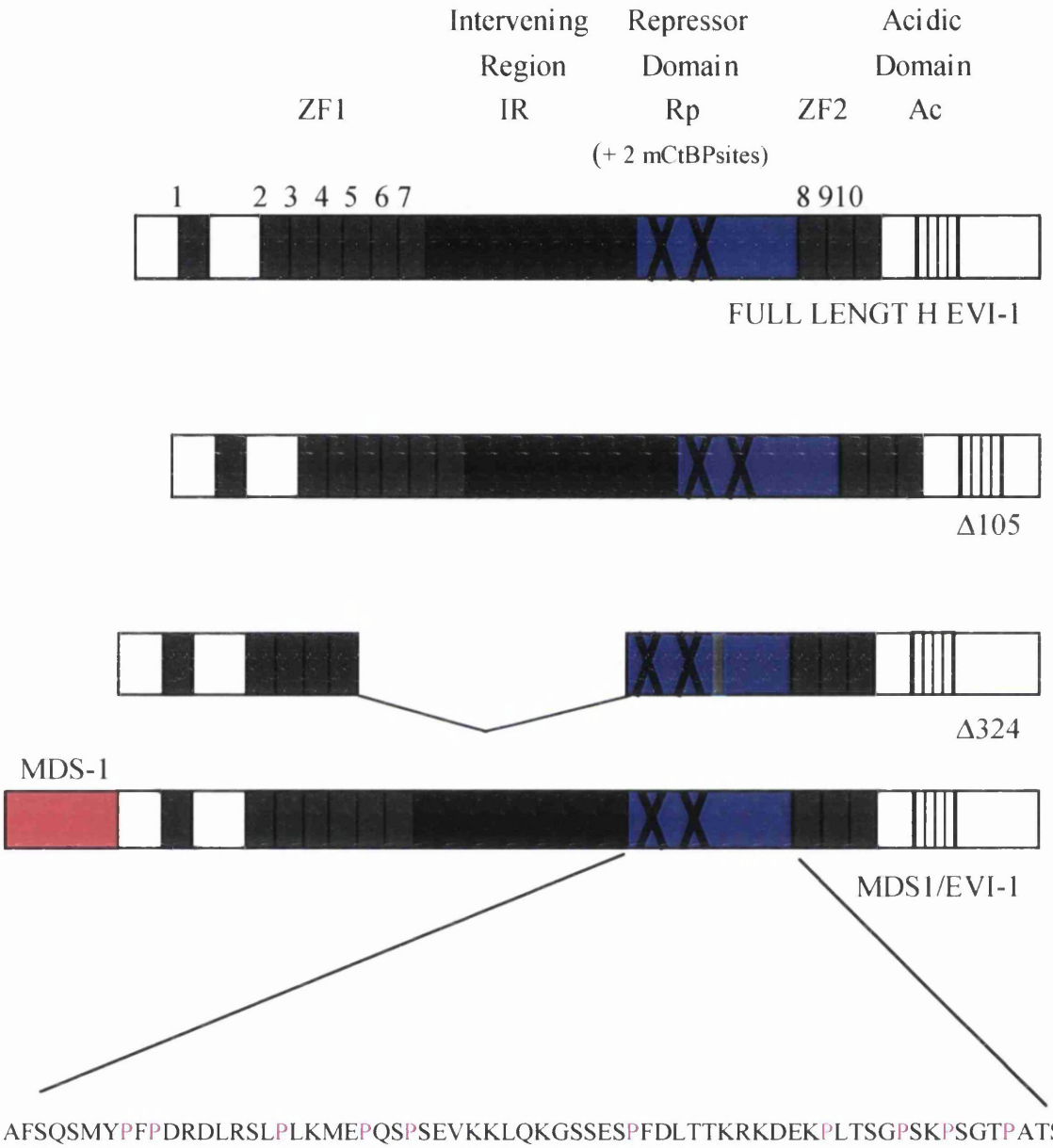

Pre-B
B-Cell
Activated
B-Cell
T-Lymphoid precursor
T-Cell
Activated
T-Cell
CFU-Eos
Eosinophil
CFU-G
Graulocyte
LTRSC
CFU-GM
CFU-M
Monocyte
CFU-Baso
Basophil
CFU-Meg
BFU-Meg
Megakaryocyte
BFU-E
CFU-E
Red Blood Cell

Figure 3: Protein Structure of Full Evi-1 and Evi-1 Alternative Splice Forms.

Grey boxes, zinc fingers (ZF1 and ZF2); Blue box, the Repressor Domain (Rp); Black/White striped box, the Acidic Domain (Ac); Black box, the Intervening Region (IR) Red Box, MDS1; Black **X**, mCtBP binding site; Light Grey Box; extra 9 amino acids in Δ324 isoform; P, proline residues within repressor domain.



AFSQSMYPFPDRDLRSLPLKMEPQSPSEVKKLQKGSSESPFDLTTKRKDEKPLTSGPSKPSGTPATS
QDQPLDLSMGSRGRASGTKLTEPRKNHVFGEKKGSNMDTRPSSDGSLQHARPTPFFMDPIYRVEK
RKLTDPLEALKEKYLRPSPGFLFHPQMAIENMAEKLESFSALKPEASELLQSVPSMFSFRAPPNTLP
ENLLRKGKERY

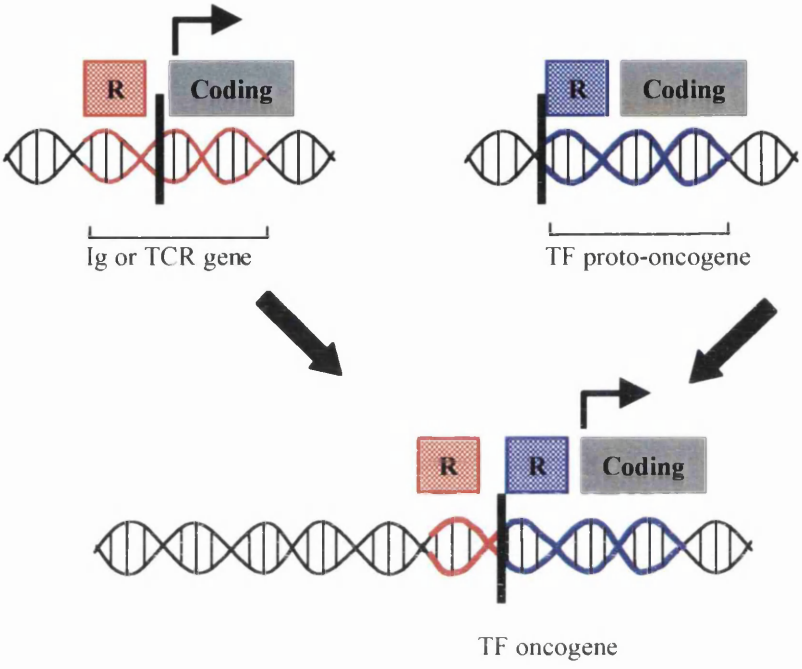
Figure 4: Two distinct mechanisms by which chromosomal translocations aberrantly activate genes encoding transcription factor

A) Recombinase error results in the activation of normally silent proto-oncogenes.

B) Breaks occurring within introns between coding regions of two transcription factors genes in separate chromosomes, create a fusion gene encoding a chimaeric protein.

(For A + B, R=regulatory region and TF=transcription factor).

A)


R
Coding
R
Coding
Ig or TCR gene
TF proto-oncogene
R
R
Coding
TF oncogene

B)

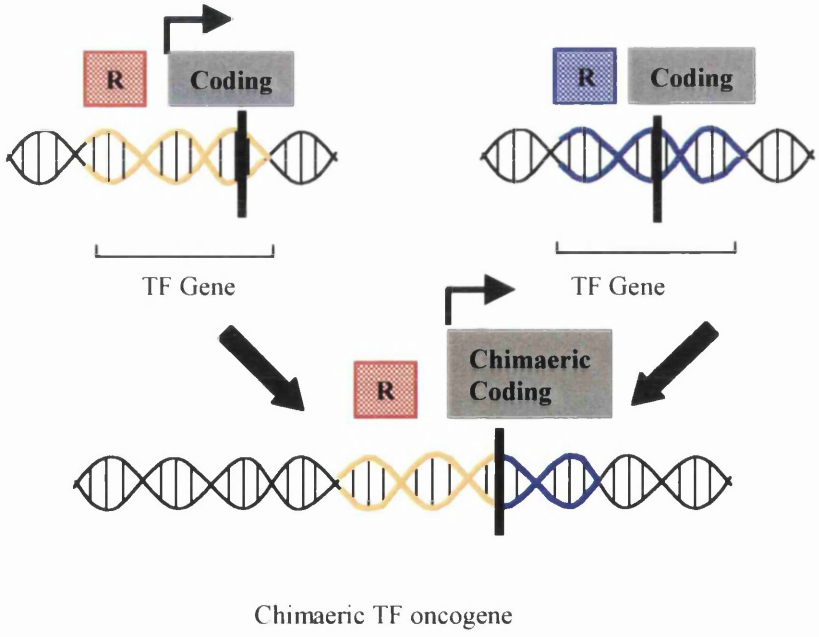

R
Coding
R
Coding
TF Gene
TF Gene
R
Chimaeric
Coding
Chimaeric TF oncogene

Table 1: Table of Fusion Genes Created by Chromosomal Translocations in Leukaemias and Solid Tumours

AML, acute myeloid leukaemia; APL acute promyelocytic leukaemia; ALL, acute lymphoblastic leukaemia; ANLL, acute non-lymphoblastic leukaemia; MDS, myelodysplastic syndrome; CML chronic myeloid leukaemia.

Tumour-Type	Cytogenetic Abnormality	Genes Involved	Protein
HAEMATOPOIETIC TUMOURS			
CML, (MDS, APL)	t(3;21)(q26;q22)	AML1/EVI-1	Drosophila runt homologue/ Multiple zinc fingers
MDS	t(3;21)(q26;q22)	AML1/EAP	Drosophila runt homologue/ Ribosomal protein L22
MDS	t(3;21)(q26;q22)	AML1/MDS1	Drosophila runt homologue/ 40% Homologue to RIZ
ANLL	t(6;9)(p23;q34)	DEK/CAN	Nuclear Protein/Nucleoporin
AML	t(8;21)(q22;q22)	AML1/ETO	Drosophila runt homologue/ Zinc Finger
APL	t(11;17)(q23;q12)	PLZF/RARα	Zinc finger/Retinoic acid receptor α
APL	t(5;17)(q32:q21)	NMA/RARα	Nucleophosmin/ Retinoic acid receptor α
APL	t(11;17)(q13;q21)	NuMA/RARα	nuclear matrix associated genes/ Retinoic acid receptor α
APL	t(15;17)(q22;q12)	PML/ RARα	Zinc finger/Retinoic acid receptor α
AML, ANLL	inv(16)(p13;q22)	MYH11/CBFβ	Smooth muscle myosin heavy chain/ Heterodimerises with AML1
ALL	t(1;19)(q23;p13)	PBX1/E2A	Homeodomain/ β HLH transcription factor
B-ALL, CML	t(9;22)(p12;q22)	BCR/ABL	Serine Kinase/Tyrosine Kinase
AML	t(9;12)(q34;p13)	TEL/ABL	ETS-related transcription factor/ Tyrosine Kinase
AML, Pre-B ALL	t(12;21)(p12;q22)	AML1/TEL	Drosophila runt homologue/ ETS-related transcription factor
ALL	t(10;14)(q24;q11)	HOX11/TCRα	Homeobox/T-cell receptor promoter
SOLID TUMOURS			
Primitive neurectodermal tumours (Ewing_s Sarcoma)	t(11;22)(q24;q12)	FLI1/EWS	ETS-related Transcription factor/Nuclear Protein
	t(21;22)(q22;q12)	ERG/EWS	ETS-related Transcription factor/Nuclear Protein
	t(7;22)(p22;q12)	ETV1/EWS	ETS-related Transcription factor/Nuclear Protein
Melanoma	t(12;22)(q13;q12)	ATF1/EWS	BZip transcription factor/Nuclear Protein

*(Adapted from Strout e al., 1997 and Sanchez-Garcia, 1997)

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Cell Culture

2.1.1.1 Cells and Reagents

Cells

Supplier: Anne Wyke, Beatson Institute for Cancer Research, Switchback Road, Glasgow G611BD.

Rat1

Supplier: Dr. A. Kilbey, Glasgow Caledonian University, Biochemistry Department, Cowcaddens Road, Glasgow G4 OBA.

Rat1 Mxneo Stables (2/7)

Rat1 FL Evi-1 Stables (3/7)

Supplier: Dr. D Baltimore, Rockefeller University, Box 161, 1230 York Avenue, New York, NY 10021.

Bosc23

Supplier: American Type Culture Collection, Rockville, Maryland 20852, USA.

293

Supplier: C. Bartholomew, Glasgow Caledonian University, Biochemistry Department, Cowcaddens Road, Glasgow G4 OBA.

Da-3

Supplier: American Type Culture Collection, Rockville, Maryland 20852, USA.

NIH3T3

Supplier: Dr. Vincent O'Brien, Beatson Institute for Cancer Research, Switchback Road, Glasgow G611BD.

Cos-7

Supplier: Harlan UK Ltd, Shaws Farm, Blackthorn, Bicerster Oxon, OX6 OTP.

CBA/Ca female mice (Primary Murine Bone Marrow Cells flushed from femurs).

Reagents

Supplier: Beatson Institute Central Services

Sterile distilled water

Sterile PBS (dulbecco "A")

Sterile PBS/1mM Edta

Supplier: Gibco Europe Life Technologies Limited, Paisley, UK.

7.5% (w/v) sodium bicarbonate

100mM sodium pyruvate

200mM L-Glutamine

0.25% Trypsin 1mM Edta.4Na

Mycophenolic Acid Crystalline

Geneticin G418 Sulphate

HT Supplement 50X

HAT Supplement 50X

Supplier: Stem Cell Technologies INC. Metachem Diagnostics Ltd. 29 Forest Road, Piddington, NN7 2DA,UK.

Pokeweed Mitogen Stimulated Murine Spleen Conditioned Medium (PWM-SCCM): prepared by incubating mouse spleen cells for one week at 37°C in RPMI 1640 and pokeweed mitogen, supplemented with bovine insulin to replace all serum components. It is not concentrated or purified.

Complete Methocel : 1% Methylcellulose in Iscoves's MDM, 15% Feotal Bovine Serum, 1% Bovine Serum Albumin, 10μg/ml Pancreatic Insulin, 200μg/ml Human Transferrin, 10^{-4} M 2-Mecaptoethanol, 2mM L-Glutamine, 50ng/mL rm SCF, 10ng/mL rm IL-3, 10ng/mL rh IL-6 and 3units/ mL rh Erythropoietin

Zapoglobin

Supplier: Sigma Chemical Co., Poole, UK.

RPMI 1640 (with NaHCO3, without Glutamine)

1X DMEM (4500mg L-Glucose, 110mg Sodium Pyruvate and Glutamine)

10X DMEM (with 4500 mg/L glucose at 1X. Without L-glutamine, folic acid and sodium bicarbonate)

α-MEM (with Ribonucleotides and Deoxyribonucleotides)

Feotal Calf Serum (European Origin)

New Born Calf Serum (Mycoplasma Screened, Virus Screened)

Horse Serum (European Origin)

Xanthine Sodium Salt

Adenine

Hepes Buffered Saline

$CaCl_2$

Polybrene

Chloroquine

Penicillin/Streptomycin Solution 100X

Supplier: Fisions Scientific Equipment, Loughborough, England.

DMSO

Supplier: Dow Corning GMBH, 65201, Wiesbaden, USA.

Vacuum Grease

Supplier: Difco Laboratories, Detroit MI 48232-7058, USA.

Agar Noble

2.1.1.2 Cell Culture Kits

Supplier: Stratech Scientific Limited, 61-63 Dudley Street, Luton, Bedfordshire, LU2 ONP.

Invisorb® Spin DNA Micro Kit III

2.1.1.3 Cell Culture Plasticware

Supplier: A/S Nunc TCS Limited, Botolph Claydon, UK.

Nunc Delta Tissue Culture Flasks (T25, T75, T150)

Nunc Cryotubes

Supplier: Beckon Dickinson Labware, Plymouth, UK.

Falcon tissue culture dishes 30mm, 60mm, 90mm and 150mm.

Supplier: Costar Corporation, Cambridge MA, USA.

6 well, 24 well and 96 well tissue culture plates

Cell scrapers

Supplier: Bibby Sterilin, Tilling Drive, Stone, Staffordshire ST15 0SA.

Sterilin petri dishes 30mm, 60mm, 90mm.

Supplier: Technical Photosystems, Cumbernauld, UK.

KodaColor Gold 400 Slide Film

2.1.1.4 Cell Culture Solutions, Media and Semi-solid Media

Cell Culture Solutions

2XHBS pH7.05 -100mls

1.6g NaCl, 0.074g KCl, 0.027g $NaHPO_4.2H_20$, 0.2g Dextrose and 1g HEPES.

Add 90mls distilled water pH to 7.05 with 1M NaOH make up to 100ml with distilled water. Filter sterilise with 0.2μm Filter.

2M $CaCl_2$- 100mls

54g $CaCl_2$

Make up to 100mls with distilled water. Filter sterilise with 0.2 μm filter.

Xanthine (40X) Add 0.2g Xanthine to 20ml 0.1N NaOH. Filter sterilise with 0.2μm filter. Aliquot, cover in foil and store at 4°C.

Adenine 12.5mg/ml

Add 0.05g Adenine to 4mls distilled water. Filter sterilise with 0.2μm filter. Store at 4°C. Use within one week.

Mycophenolic Acid (250X)

Add 250mg to 40mls 0.1N NaOH. Filter sterilise with 0.2μm sterilising cup. Aliquot, cover in foil and store at -20°C.

Chloroquine made up at 100mM

Add 1.29g Chloroquine to 25mls distilled water. Filter sterilise with 0.2μm filter. Aliquot, cover in foil and store at -20°C.

IL-3 at 0.1μg/ml

5g in 10 ml Filter Sterile. Filter sterilse with 0.2μm filter. Aliquot and store at -20°C.

G418 (400mg/ml)

Add 5g to 12.5ml PBS. Filter sterilise with 0.2μm filter. Aliquot and store at -20°C.

Cell Culture Media and Semi-Solid Media

Bosc23 HAT-supplemented growth medium

1X Dulbecco's Modified Eagles Medium (1 X DMEM), 10% FCS, 2mM L-Glutamine, 1X HAT Supplement, 250μg/ml Xanthine (1X), 25μg/ml Adenine and 50μg/ml Mycophenolic Acid (1X).

Bosc23 HT-supplemented growth medium

1X DMEM, 10% FCS, 2mM L-Glutamine, 1X HT supplement and 25μg/ml Adenine.

Bosc23/Bone marrow co-cultivation medium

α-Mem, 25% horse serum, 2mM L-Glutamine, 1X HT supplement, 25μg/ml adenine and 250 μg/ml xanthine.

Rat1 fibroblast culture medium

1X DMEM, 5% NBCS and 2mM L-Glutamine.

Rat1 Transformation Assay Semi-solid Media

Base Agar—100mls

1X DMEM (made from 10X DMEM concentrate), 10% FCS, 0.375% Sodium Bicarbonate Solution (stock @ 7.5%), 0.1mM Sodium Pyruvate, 2mM L-Glutamine, 3% Agar, 1 x Penicillin /Streptomycin - Made up to 100mls with distilled sterile water.

Top Agar = 1:1 Base Agar:Cells (in 1x DMEM/20%FCS).

Cos7 and NIH3T3 culture medium

1x DMEM, 10% FCS and 2mM L-Glutamine.

Da-3 culture medium

RPMI 1640, 10% FCS, 4mM L-Glutamine and 0.1ng/ml IL-3.

Trypsin solution for routine passing

0.25% Gibco trypsin in sterile PBS/1mM EDTA

2.1.2 Cell Culture Machinery

Supplier: Beatson Institute Workshop, Beatson Institute for Cancer Research, Switchback Road, Glasgow, G115AY.

Cloning Rings

Supplier: ORME Technologies, Whitbrook Way, Middleton, Manchester M24 2RH.

Heraus

37°C 10% CO_2 incubator

37°C 5% CO_2 incubator

33°C incubator

Supplier: The Baker Company, Philip Harris Scientific, Lichfield, Staffordshire W514OEE, UK.

Class II Type A Tissue Culture Hood

Supplier: Olympus Optical Company Ltd, Great Western Industrial Park, Dean Way, Middlesex UB2 48D.

Microscope plus associated camera

Supplier: Leica Camera, Knowhill, Milton Keynes MK58LB.

Inverted Microscope

Supplier: Schärfe System GMBH, Emil-Adolff Straβe 14, D-72760 Reutlingen

Schärfe System Casy I - Coulter Counter

2.1.3 Antisera and associated materials

Supplier: Amersham International, Little Chalfont, UK.

anti-mouse/horse radish peroxidase conjugate

anti-rabbit/horse radish peroxidase conjugate

GAL4 monoclonal

Supplier: Dr. C. Bartholomew, Glasgow Caledonian University, Biochemistry Department, Cowcaddens Road, Glasgow, G4 OBA.

Evi-1 anti-serum, mouse polyclonal (1806)

2.1.4 Protein Electrophoresis and Immunoblotting

2.1.4.1 Protein Electrophoresis and Immunoblotting Reagents

Supplier: Amersham, Little Chalfont, UK.

High Molecular Weight Rainbow Markers (14.4kDa to 220kDa)

Hybond ™ N^+ nylon membrane

Enhanced Chemoluminescence Reagent Solution

Supplier: Pierce, PO BOX 117, Rockford, Illinois, 61105, USA.

Coomassie Blue Protein Assay Reagent

Supplier: Sigma Chemical Co., Poole UK.

Micro BCA/ $CuSO_4$ Protein Assay Kit (Bicinchoninic Acid Solution including Copper II Sulphate and Protein Standards)

TEMED (N,N,N',N'- Tetramethylethylenediamine) 0.1% (w/v) Ponceau S Solution in 5% acetic acid)

Bovine Serum Albumin (BSA), Fraction V

Supplier: Severn Biotech Ltd, Kidderminster, UK.

Design-a-gel 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide solution

Supplier: Technical Photosystems, Cumbernauld, UK.

Fuji RX X-Ray film

Supplier: Whatmann, Maidstone, UK.

3mm filter paper

2.1.5 Protein Extraction and Processing

Supplier: Sigma Chemical Co., Poole UK.

All Protease Inhibitors

Protein A sepharose beads

Supplier: Dow Chemical Company Ltd., Sully, Penarth

Vale of Glamorgan, South-Wales, CF64 5YB.

Saran Wrap

Supplier: Swann Morton Limited, Owlerton Green, Sheffield S62BJ.

Disposable Scalpels

Supplier: Asda, Saughs Road, Robroyston, Glasgow City Centre,

Strathclyde, G331P.

Marvel

2.1.5.1 Immunoblotting Solutions and Buffers

Group A protease inhibitors— Stock Solutions all stored at -20°C.

50mM PMSF (100X) Add 0.87g to 100ml isopropanol

50mM Benzamidine (100X) Add 0.78g to 100ml distilled water

1M β-glycerophosphate (100X) Add 21.6g to 100ml distilled water

1mM Sodium Orthovanadate (20X) Prepare 100mM Stock: Add 1.83g to 100ml distilled water. Dilute to 1mM with distilled water, pH to 8.0 and boil in a water bath until yellow colour disappears.

Group B protease inhibitors

1mg/ml Leupeptin (1000x) in distilled water

Pepstatin A (1000X) in methanol.

Whole Cell Extract Lysis Buffer

20mM Hepes, 5mM EDTA, 10mM EGTA, 1mM DTT, 0.4M KCL, 0.4% Triton X-100, 10% Glycerol and 5mM NaF. Add protein inhibitors 5μg/ml Aprotinin, 0.01mg/ml Okadaic acid, 5μg/ml Pepstatin A, 5μg/ml leupeptin, 100mM PMSF and 50mM Benzamidine

SDS-PAGE Electrophoresis Gel Recipe

SDS Resolving Gel	SDS-PAGE Stacking Gel	(for 7.5% Gel)
Acrylamide/Bis (30:8)	10ml	3.2ml
1M Tris pH8.8	15ml	------
10% SDS	0.4ml	0.2ml
dH2O	15ml	14ml
1M Tris pH6.8	------	2.5ml
10% Ammonium persulphate	375μl	200μl
temed	20μl	20μl

Protein Electrophoresis Tank Buffer (10X Concentrate)

1M Tris

1M Glycine

2% (w/v) SDS

Semi-dry transfer buffer for immunoblotting

48mM Tris

39mM Glycine

0.037% (w/v) SDS

20% methanol

Immunoblot Wash Buffer TBST (10X concentrate)

Tris 24.2g/2l

NaCl 175.3g/2l

Tween-20 10ml/2l

pH to 8.0 with concentrated HCL

PAGE/SDS 2x Sample Buffer

Tris/HCL pH6.8	1.3ml
Glycerol	2ml
20% SDS	2.5ml
β-Mercaptoethanol	800ml
dH_20	4.2ml

add a pinch of bromophenol blue powder.

Immunoblot Stripping Buffer

0.2M Glycine pH2.5

1% (w/v) SDS

2.1.5.2 Protein Electrophoresis and Immunoblotting Equipment

Supplier: Beatson Institute Workshop

Semi-dry Blotting Apparatus

Supplier: Genetic Research instrumentation Dunmow, UK.

Atta protein electrophoresis apparatus

2.1.6 Plasmid Preparation Solutions and associated materials

2.1.6.1 Bacterial Media and antibiotics

Supplier: Difco Laboratories, Detroit MI 48232-7058, USA.

Bactotryptone

Bacto Yeast Extract

Supplier: Gibco Europe Life Technologies Limited, Paisley, UK.

Luria-Broth Base

Luria-Broth Agar

Supplier: Sigma Chemical Co., Poole, UK.

Ampicillin

2.1.6.2 DNA Analysis— Materials

Supplier: Boehringer Mannheim UK., Lewes, East Sussex, England.

Calf Intestinal phosphatase (1U/μl)

Supplier: BDH

Bromophenol Blue

Agarose Gel Loading Buffer

Supplier: Biowhittaker UK Limited, Biowhittaker House, Berkshire, RG41 2PL.

All Restriction Enzymes + 10X Restriction Buffers

T4 DNA polymerase

Supplier: Sartorius AG, 37070 Goettingen, Germany

Collodium bags (Dialysis Tubes)

Supplier: Vernon-Carus Limited, Penwortham Mills, Preston, Lancs, England.

Cotton Mesh

Supplier: Bioline, 16 The Edge Business Centre, London NW2 6EW, UK.

Biotaq DNA Polymerase

Supplier: Promega, Delta House, Chilworth Research Centre, Southampton SO16 7NS.

DNTPs

T4 DNA Ligase (10U/μl) + 10X Buffer

Supplier: Sigma Chemical Co., Poole, UK.

1Kb Ladder

Ethidium Bromide tablets

2.1.6.3 DNA Analysis Kits

Supplier: Qiagen Limited, Boundary Court, West Sussex, RH10 2AX.

Qiagen Midiprep

Qiagen Maxiprep

Qiaquick Gel Extraction

Qiaquick PCR Purification Kit

2.1.6.4 DNA Analysis Solutions

Solution I

50mM Glucose

25mM TrisHCL (pH 8.0)

10mM EDTA

Solution II

0.2N NaOH

1% SDS

Solution III

To 300ml Potassium Acetate add 57.5ml Glacial Acetic Acid and 142.5ml distilled H_2O.

The resulting solution is 3M with respect to potassium and 5M acetate.

Dialysis Solution (100mM Tris/1mM Edta)

20ml Tris/ 4ml Edta in 2L Distilled Water.

Luria Broth (1L)

25gm of Luria Broth Base is added to 1L of Ultra Pure Milli-Q water. This solution is autoclaved.

Luria Agar Plates

37gm of Luria Agar is added to 1L of Ultra Pure Milli-Q water. The agar is autoclaved and allowed to cool to 50°C. The appropriate antibiotic is added, generally ampicillin at 50μg/ml, and the agar plates are poured into 90mm Sterilin dishes.

Terrific Broth (1L)

To 900ml of Ultra Pure Milli-Q water we added 12g Bactotryptone, 24g Bacto-yeast Extract and 4ml Glyerol. This solution is autoclaved , cooled to 50°C then 100ml of a sterile solution of 0.17M Kh_2PO_4 0.72 M K_2HPO_4 is added.

SOC medium (1L)

To 950ml of Ultra Pure Milli Q water add 20g Bactotryptone, 5g Bacto-Yeast Extract and 0.5g NaCl. Dissolve. Add 10ml of a 250mM solution of KCl. pH to 7.0. Adjust the volume to 1L with Ultra Pure Milli Q water. Sterilise by autoclaving. Allow solution to cool to 50°C then all 20ml of a sterile 1M solution of glucose. Before use add 5ml of a sterile 2M $MgCl_2$ solution.

TAE 50X (1L)- Stock

Add 242g Tris Base to 57.1 Glacial Acetic Acid and 100ml 0.5M EDTA make up to 1000ml with distilled water.

2.1.6.5 DNA Analysis Machinery

Supplier: Kontron Instruments, Blackmore, Croxley Business Park, Hertfordshire WD1 8XQ.

Centrikon Ultra-Centrifuge T-2050

Supplier: Kendro Laboratory Products Ltd., Hertfordshire CM23 5G2.

Sorvall Centrifuge including SS-34 Rotor

Beckman Spectrophotometer

Beckman Centrifuge J2-21 including rotors JLA-10.5 and JA-12

Supplier: Bibby Sterilin, Tilling Drive, Stone, Staffordshire ST15 0SA.

37°C Orbital Shaker

Supplier: Perkin Elmer Analytical Instruments, Chalfont Road, Seer Green HP9 2FX.

Perkin Elmer 9600 (BIGD) Thermal Cycler

Supplier: MJ Research INC, Watertown, MA 02172, USA.

PTC-100 Programmable Thermal Controller

Supplier: Bio-rad Laboratories Ltd., Bio-rad House, Hemel Hampstead, Hertfordshire HP2 7TD.

Gel Electrophoresis Apparatus including Power packs

2.1.7 Cytospin Materials

Supplier: BDH Laboratory Supplies, Poole, Dorset, BH15 1TD.

Glass slides

Giemsa

Filters

Sample holders

2.1.7.1 Cytospin Solutions

10% Giemsa in PBS

10ml Giemsa /90ml PBS

2.1.7.2 Cytospin Machinery

Supplier: IEC Harwell Road, Poole BH1 70BD, UK.

Cytocentrifuge

2.1.8 Flow Cytometry Reagents and Associated Materials

Supplier: Boehringer Mannheim Lewes, UK.

RNaseA

Supplier: A/S Nunc TCS Limited, Botolph Claydon, UK.

FACS tubes

Supplier: Sigma Chemical Co., Poole, UK.

Propidium iodide

2.1.8.1 Flow Cytometry Solutions

Propidium Iodide (PI) Stain

Prepare a 1mg/ml PI stock solution in PBS and a 10mg/ml pre-boiled solution of RNaseA. To give a final concentration of 10μg/ml PI and 25μg/ml RNaseA in PBS: add 100μl of PI stock and 250μl RNaseA stock to 10ml PBS.

2.1.8.2 Flow Cytometry Machinery

Supplier: Beckton Dickinson, Plymouth, UK.

Beckton Dickinson FACScan Flow Cytometer

Modfit Software

2.1.9 Radioactivity

Supplier: Amersham International Plc., Amersham, Bucks, UK.

^{14}C-Chloramphenicol

2.1.10 General Chemicals

Supplier: Sigma Chemical Co., Poole, UK.

Calcium Chloride

Hepes Buffered Saline

Beta-mercaptoethanol

Hydrochloric acid

Sodium Hydroxide

Ethanol

Butanol

Methanol

Tris

EDTA

EGTA

Triton-X-100

Orange G

Ficoll

Supplier: Gibco Europe Life Technologies Limited, Paisley, UK.

1M Hepes pH 7.5

50X HAT supplement

50X HT supplement

mycophenolic acid

SDS Lauryl Sulfate

Sodium Chloride

Magnesium Chloride

Potassium Chloride

Glacial acetic acid

Supplier: BDH Laboratory Supplies, Poole, UK.

Ammonium peroxodisulfate (APS)

Glycine

All other chemicals not listed where from Fisher Scientific.

2.1.11 Other General-Use Laboratory Machinery

Supplier: Fisher

Whirlimixer

Supplier: Anachem House, Charles Street, Luton, Bedfordshire LU2 OBE.

Heat Block

Supplier: ORME Technologies, Whitbrook Way, Middleton, Manchester M24 2RH.

Stuart Scientific Magnetic Stirrers

Grant Water Bath

Heraus Microfuge

2.2 Methods

2.2.1 Bone Marrow Colony Assays

2.2.1.1 Production of Virus

Adherent human Bosc23 cells (Pear et al., 1993) are selected and maintained in DMEM (2mM L-Glutamine, and 10% Fetal Calf Serum) supplemented with hypoxanthine-aminopterin-thymidine or HAT, 250 μg/ml xanthine, 25 μg/ml adenine (Sigma) and 50μg/ml mycophenolic acid. Retroviral producer cell lines are generated in Nunc T-25 flasks each containing $2x10^6$ Bosc23 cells which are transfected with 4μg of DNA

(p50MXneo, p50MEvi-1Flneo or Evi-1 mutant vectors) using the calcium phosphate method. For each $25cm^2$ monolayer of cells to be transfected the calcium phosphate-DNA coprecipitate is prepared as follows: 4μg DNA is made up to 437.5μl with ultra pure MilliQ distilled water then 62.5μl 2M $CaCl_2$ is added. This mixture is vortexed and then added dropwise to 500μl 2XHBS whist bubbling the DNA mixture with air. The DNA is left to precipitate for 20 minutes. This 1ml mixture is added to 5ml of medium in the $25cm^2$ flask of cells. During transfections Bosc23 cells are supplemented with HT, μg/ml xanthine, 25μg/ml adenine and penicillin/ streptomycin.

2.2.1.2 Retroviral-Mediated Gene Transfer into Murine Bone Marrow

Bone Marrow cells are isolated from the femurs of CBA/Ba mice. The haematopoietic cells are flushed from each femur with 10ml of α-MEM using a 23-Gauge needle. Each universal, containing bone marrow from one femur, is spun down at 1000rpm for 5 minutes. The cells are then re-suspended in co-cultivation medium (α-MEM, 25% horse serum and 2mM L-Glutamine supplemented with HT, 25 μg/ml adenine and 250 μg/ml xanthine). The bone marrow cells are now carefully added to T25 flasks (one femur/flask) of pre-irradiated Bosc23 producer cells. This co-cultivation is left for 72 hours. After infection, the cells (bone marrow plus dead feeders) are harvested and washed in α-MEM. After determination of the total viable nucleated cell count by addition of zapoglobin to cells, 8000 cells and 20000 cells are plated out into growth factor supplemented methylcellulose (see section 2.1.1.1) for each infection. The plates were incubated at 37°C 10% CO_2 and haematopoietic colonies were scored at 7-10 days.

2.2.1.3 Molecular Analysis of Individual Bone Marrow Colonies

Polymerase chain reaction (PCR) is used to assess proviral integration of individual colonies isolated from methylcellulose culture. To avoid contamination all the following procedures were carried out within an UV-irradiated sterile environment. Individual colonies are identified and isolated into microfuge tubes and placed on ice. Immediately after colony isolation, DNA was extracted from colonies using a DNA extraction kit. Two PCR reactions are carried out using external primers complementary to the neo cassette (5'-3') and to the p50Mxneo vector itself (3'-5) and nested primers to the neo cassette only. The PCR reaction contained lysate/template, 1x Buffer (Promega), 3mM $MgCl^2$, 2mM DNTP's, 0.35ng of each primer (140ng/μl) and 2.5 units Taq (Promega), in a 50 μl reaction. Cycling conditions used an initial denaturation of 94 for 2 minutes: 30 cycles of 94C for 1 minute; 55°C for 1 minute and 72° for 30 seconds; and a final extension of 72°C for 7 minutes. The PCR-amplified products were analysed by gel electrophoresis.

2.2.1.4 Cytospin slide preparation

For each colony type, 1-10 colonies are picked and suspended in 200μl of ice cold PBS. Slide holders are prepared with slide and filter. All 200μl of each cell suspension is placed into the sample holders. The cells are spun onto the slide in a cytocentrifuge at 500rpm for 5 minutes. The slide is air dried for 5 minutes, fixed in 100% methanol for 10 minutes the stained in 10 % Giemsa in PBS for 10 minutes. The stain is then displaced with running water. Slides are air dried, then mounted with DPX and a coverslip.

2.2.2 Cell Cryopreservation and Recovery

Cells are grown to 70% confluence in T175 flasks. The cells are harvested, counted and resuspended at $5x10^6$ cells/ml in 10% DMSO in Fetal Calf Serum. The cells are added 1ml Nunc Cryotubes, wrapped in cotton wool (to slow freeze) and placed at -70C. The next day the cells are transferred into a Liquid nitrogen storage vessel.

When recovering cells from liquid nitrogen, the cells are thawed in water at 37°C within a large, covered plastic vessel. The cells are completely removed from the vial using a 19 gauge needle and placed into a T25 tissue culture flask. 5mls of the appropriate medium is added to the cells drop by drop. The flasks are placed at the appropriate incubation.

2.2.3 NIH3T3 Focus-formation assays for retroviral titre estimation

$CaPO^{4-}$ transfections in Bosc23 are carried out as described in Section 2.2.1.1. Medium containing virus is harvested 48 hours post transfection and filtered through a pre-wetted 0.2μm plastic filter.

NIH3T3 cells are plated in 24 well plates at 8000 cells per well in DMEM (10% FCS, 2mM L-Glutamine and 8μg/ml polybrene) twenty-four hours prior to harvesting of virus. Duplicate wells are seeded for each virus tested.

Serial dilutions of virus ranging from 1/10 through 1/31250 are carried out for each virus. Two days after the addition of virus to NIH3T3 cells the medium is replaced with DMEM containing 800μg/ml G418. After 10 days in this selection media is removed, the cell foci are washed in PBS, fixed and stained in methanol and Giemsa respectively and then counted.

2.2.4 Rat1 Transformation Assay

2.2.4.1 Production of Selectable Population or Clones

Bosc23 cells are transfected as described above (Section 2.2.1.1). Forty-eight hours post transfection and after a sixteen-hour collection time in 2mls of medium, virus is harvested. The viral supernatant is filtered through a pre-wetted 0.45μm plastic filter and all 2mls are added to a 60mm dish with adherent Rat 1 cells at $5x10^5$ cells/dish plated 12-24 hours previously. The next day the Rat 1 cells in the 60mm dish are split into two 90mm dishes and placed in a 5% CO_2 incubator. Twenty-four hours later the appropriate selection is added to the 90mm dishes (1mg/ml zeocin and/or 1mg/ml G418). The cells are selected for a further 7-10 days and emerging clones are either grown to confluence as a cell population or they are single cell cloned by ring cloning. The cells are grown up and whole extracts prepared to assess protein expression of introduced vectors.

2.2.4.2 Plating Cells in Soft Agar

After determination of protein expression by Western Blotting the cell lines of interest are plated in agar. The assay is set up using two layers of agar medium. The underlayer consists of a 0.6% agar/DMEM mixture (see section 2.1.1.4). The top layer is 0.3% agar/DMEM (see section 2.1.1.4) and contains the cells plated at 10^3 cells per 60mm dish. The plates are left three to five weeks in a 10% CO^2 humid incubator. After this time anchorage independent colonies over 0.1 cm are scored.

2.2.5 Protein Analysis

2.2.5.1 Preparation of Whole Cell Extracts

Plates are rinsed in ice cold PBS and drained for 20 minutes at 4°C. Residual PBS is removed and lysis buffer is added to each plate (add 75μl /60mm dish or 100μl/90mm dish). Plates are then scraped, drained for 5 minutes and the lysis buffer is transferred to a microfuge tube. Extracts are spun for 20 minutes at 13K, 4°C. The supernatant is transferred into a screw cap tube and protein concentrations are determined using Coomassie Blue. Extracts are stored at -70°C.

2.2.5.2 Western Blotting

Protein Electrophoresis

The table in section 2.1.5.1 shows the recipes for 7.5% and 10% acrylamide gels. The gel apparatus is set up using 2mm gel plates and spacers. The resolving gel is poured and approximately 5mm of water saturated butanol is laid on top. After the gel has set, the

butanol is poured off, distilled water is used to rinse away any last traces and the gel surface is blotted with Whatmann 3mm paper. The stacking gel is the poured and a 2mm comb is inserted. After the stack has set the comb is removed and the gel is removed from the gel former and placed into a tank. The tank is then filled with 1x Running Buffer (see section 2.1.5.1).

The samples are prepared by adding an equal volume of extract to 2X sample buffer. The samples are loaded into the wells along with pre-stained size markers. The gel is run at 250V for 2hours. When electrophoresis is complete the gel is removed and washed in semi-dry blot transfer buffer in 20% methanol. Samples are then transferred to ECL membrane using a semi-dry blotter. The gel, ECL membrane and 12 pieces of 3mm Whatmann are subsequently soaked in semi-dry transfer buffer and arranged in the blotter as follows; 6 pieces of filter paper, gel, membrane and 6 pieces of filter paper. Transfer of proteins to ECL membrane occurs at 20V over a 2 hour period. After transfer the blot is blocked overnight in 5% Marvel in TBST(see section 2.1.5.1).

Antibody incubations and detection by ECL

The blot is washed for 15 minutes in TBST. The TBST is decanted and 20ml of 5% Marvel/TBST with the appropriate primary antibody is added. The blot is now shaken in the primary antibody for typically 1hour at room temperature. The blot is then rinsed and washed in TBST for 1hour with fresh buffer changes every 15 minutes. The blot is now shaken in 20mls of 5% Marvel/TBST with the appropriate secondary antibody - again typically for 1hour at room temperature. The blot is rinsed and washed as after the

primary antibody. The TBST is then drained off on Whatmann 3mm paper and ECL detection reagents are added according to the manufacturers instructions. The filter is wrapped in saran wrap and exposed to x-ray film.

Stripping immunoblots to remove anti-sera

To remove proteins from ECL membranes blots are washed in immunoblot stripping buffer (0.2M Glycine pH2.5/1% (w/v) SDS) for 1hour. They are then washed for a further 2hours with fresh stripping buffer changes every 15 minutes. Blots are blocked overnight in 5% Marvel/TBST. These blots are washed in TBST for 15 minutes and are now ready to reprobe with antibody.

2.2.7 Bacterial Cell Culture

2.2.7.1 Host Strains

E.Coli strains XL1 or DH5a were obtained from Beatson institute stocks. Bacterial strains were grown in Luria-Broth at 37°C with good aeration.

2.2.7.2 Competent Cells

Competent bacterial cells were obtained from Gibco.

2.2.7.3 Transformation of Bacterial Cells

Competent cells were thawed slowly on ice and 100μl transferred to a pre-chilled 15ml flacon tube. 1μl of plasmid or an entire DNA ligation reaction was pipetted into the cells

mixed gently and incubated on ice for 30 minutes. 1ml of Luria-Broth was then added and the cells were incubated for 1hour in a shaker at 220rpm. The cells were heat shocked at 42°C for 2 minutes. 200μl L Broth and 5μl cells were then spread onto 1.5% (w/v) agar Luria-Broth plates containing the appropriate anti-biotic at 37°C overnight.

2.2.7.4 Glycerol Stocks

E.Coli strains containing plasmids were stored as glycerol stocks. Stationary cultures were mixed with an equal volume of sterile glycerol and stored at -20°C.

2.2.8 Nucleic Acids: DNA Analysis

2.2.8.1 Mini Plasmid Preparations

Bacterial colonies were picked using a sterile tooth pick and grown up overnight in 5ml of L broth medium, containing the appropriate antibiotic, in an orbital shaker. Glycerol stocks were prepared by adding 0.5ml of this overnight culture to 0.5ml sterile glycerol. These stocks are stored at -20°C. 3 ml of the remaining cells was harvested by centrifugation at 3000 rpm for 5 minutes/4°C in a microfuge. The cell pellet was resuspended in 200µl Solution I (50mM Glucose, 25mM Tris/HCL pH 8.0, 10mM EDTA) and incubated on ice for 10 minutes. 400μl of Solution II (0.2N NaOH, 1% SDS) was then added, the solution mixed very gently and incubated for 5 minutes on ice. 300µl Solution III (3M Potassium Acetate, 11.5% glacial acetic acid) was added , the tube was inverted for 15 seconds and incubated on ice for a further 10 minutes. After incubation the mixture was spun at 14000rpm for 10 minutes/4°C. 500 μl of the cleared supernatant

supernatant was transferred to a fresh eppendorf and1ml isopropanol was added. The tube was then placed on dry ice for 10 minutes then centrifuged on full speed for 10 minutes/4 °C. The pellet was then resuspended in 100μl 1 X TE and 0.3 M NaOAc. For extraction, 50 μl of phenol/chloroform/isoamyl alcohol (24parts/24parts/1 part) was added, the tube was vortexed then spun in at 14000rpm for 5 minutes. The aqueous phase was then transferred to a fresh eppendorf. 300μl absolute ethanol was added to precipitate plasmid DNA and this was left at -20°C for 20 minutes then spun at 14000rpm for 10 minutes/4°C. The pellet was resuspended in 1XTE ands RnaseA (0.01μg/ml). Restriction digests were carried out to analyse the new DNA.

2.2.8.2 Large Scale Plasmid Preparations

4.5ml of overnight culture was inoculated into 500ml of Terrific Broth Medium, containing the appropriate antibiotics, and grown overnight in an orbital shaker at 37°C overnight. The cells were harvested by centrifugation at 5000rpm at 15 minutes/4°C. The supernatant is discarded, the pellet resuspended in 10ml ice cold solution I. The mixture was beaten until frothy and cloudy then left on ice for 5 minutes. 20ml solution II was added. The tubes were gently rolled then incubated on ice for 10 minutes. Then 15ml solution III was added, the tubes inverted for 15 seconds and then left on ice for 15 minutes. The lysate was then cleared of precipitated SDS, proteins, membrane, and chromosomal DNA by spinning at 5000 rpm for 15 minutes at 4°C and then pouring the resultant supernatant through several layers of cotton mesh into a 50ml falcon tube. 0.6 volumes of isopropanol were then added at room temperature to precipitate the DNA.

The tubes were mixed and incubated at room temperature for 30 minutes. After incubation the tubes are spun at 4000rpm for 30 minutes /room temp. The supernatant is discarded and the pellet was resuspended in 8ml of TE (100mM Tris/HCL pH 8, 1mM EDTA). 8.8g of Caesium chloride and 800μl of ethidium bromide (at 10mg/ml) was added to the tube. The solution was transferred to an ultracentrifuge tube and spun for 24 hours at 55000rpm at room temperature. Plasmid bands were removed with a needle and syringe and transferred to a fresh tube. 8ml of TE were added along with 0.4mls of ethidium bromide and 8.4g Caesium Chloride. Again this solution is transferred to an ultracentrifuge tube and spun for a further 24 hours at 55000rpm/ room temperature. The ethidium bromide is removed from the DNA solution by butan-1-ol extraction. When the ethidium is completely remove and the butanol layer removed, the DNA is added to 4 volumes of distilled water. 2.5 volumes of ethanol are then added. The preps were left overnight at -20°C for precipitation of plasmid DNA to occur. The tubes are spun down at 4000rpm for 30minutes/ room temperature. The supernatant was removed, the pellet air dried and the resuspended in 0.5ml Milli Q ultra-pure water. DNA is then dialysed in TE (100mM Tris/HCL pH 8, 1mM EDTA) at 4°C overnight.

2.2.8.3 Optical Density Analytical Measurements

DNA is diluted 1:200 in dH_20 with a total volume of 1ml. This is mixed then transferred to a quartz spectrophotometer cuvette. The spectrophotometer is blanked with 1ml of H_20. The optical density of the sample is then read at a wavelength of 260nm. The concentration of DNA, in micrograms per microlitre of sample is 10 times the OD

reading. To assess the purity of the sample, the second reading is taken at 280nm. If the ratio of OD at 260nm to OD at 280nm is approximately 2 we can be quite confident the absorption is due to nucleic acids. If the ratio is lower than 1.7, the sample is reprecipitated by ethanol precipitation (see section 2.2.8.6).

2.2.8.4 Restriction Digests

Restriction digests of plasmid DNA were carried out with an appropriate amount of restriction enzyme in its respective buffer as recommended by the supplier. Typically, plasmid concentrations of 2μg were digested in a total of 30μl with 5-10 units of enzyme per μg DNA in 1Xbuffer for 3-4 hours at the appropriate temperature (usually 37°C). The total volume of reaction was increased for larger concentrations of DNA. Reactions were terminated by the addition of $1/10^{th}$ volume of agarose gel loading buffer.

2.2.8.5 Gel Electrophoresis

Typically 0.8% agarose gels were made to resolve restriction enzyme-digested DNA. 1.6g of agarose was added to 200ml 1XTAE. The solution was then heated in a microwave on high for 5 minutes then placed on a stirring block to cool. When hand hot, 0.5μg/ml ethidium bromide was added and the solution is poured into an appropriate gel former. Gels are set at room temperature and then transferred to an electrophoresis tank containing 1XTAE. Samples containing $1/10^{th}$ volume agarose gel loading buffer were loaded in separate wells and run at 40-120V depending on the size of fragments and time restrictions. The molecular weight standard used was a 1Kb ladder. The DNA bands were visualised using an UV source and then photographed.

2.2.8.6 Re-Precipitation of Nucleic Acids

Generally, 2.5-3 volumes of an 9.5% ethanol/ 0.12M sodium acetate solution was added to the DNA sample in a microcentrifuge tube, which was incubated at -20°C overnight. The sample is then centrifuged at full speed (13000 rpm) in a microcentrifuge for 15 minutes at 4°C. The supernatant was then decanted and the tube inverted on a paper towel. The DNA pellet is then rinsed in 80% ethanol. After a second centrifugation, the supernatant again is discarded, and the DNA pellet is dried in a Speedi-Vac for 10 minutes. The dried DNA is dissolved in TE pH 7.5.

2.2.8.7 Purification of DNA Fragments from Agarose Gels

DNA fragments were excised from agarose gels and placed into eppendorf tubes. The DNA from each fragment was isolated using the Qiagen DNA Gel Purification Kit according to the manufacturers protocol. After purification the quantity and quality of the purified fragment(s) were assessed by agarose gel electrophoresis.

2.2.9 Subcloning of DNA

2.2.9.1 Preparation of Vector DNA

Plasmid vectors were digested with the appropriate restriction enzyme and at the end of the reaction 1μl of calf intestinal alkaline phosphatase (CIAP) was added and the reaction incubated for a further 30 minutes. The vector was gel purified with a Qiagen gel purification kit.

2.2.9.2 Generation of Inserts

Inserts were generated by either restriction enzyme digestion of plasmid DNA or PCR-generated DNA. Either way, the resultant fragments were resolved by agarose gel electrophoresis and purified, as above.

PCR DNA was generated using this protocol:

In each 100μl reaction:

10ng Template DNA, 5U Pfu polymerase, 1X Buffer, 200μM dNTPs, 40pmoles of each oligonucleotide. Amplification was achieved in a Perkin-Elmer Cetus thermal Cycler by 25 cycles of 1minute at 94°C, 1minutes at 55°C and 8 minutes at 72°C followed by a final 10 minutes at 72°C extension time.

2.2.9.3 Ligation of DNA Fragments into Plasmids

The purified vector and insert DNA were digested and included in the following reaction:

20ng vector, 5 molar excess of DNA insert/s, 1μl Ligation Buffer (Stock is 10X=300mM TrisHCL pH7.8, 100mM MgCl2, 100mM DTT and 10mM ATP) and 1μl T4 Ligase made up to 10μl with distilled water.

The reactions were incubated at 16°C overnight. Reactions were stopped by a heat shock of 65°C for 5 minutes.

2.2.10 Sequencing Reactions

Big Dye Terminator Sequencing (on ABI 373A)

DNA (typically 500ng double-stranded DNA, 100ng single-stranded DNA or 90ng PCR product DNA) is mixed with 3.2pmoles primer and the volume is made up to 12μl with Milli-Q grade water. 8μl of reaction premix is added and the PCR is carried out using a Perkin Elmer 9600 (BIGD) with the following conditions: preheat to 96°C, 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes and soak at 4°C. The DNA was precipitated with 2μl of 3M Sodium acetate and 50μl absolute ethanol. This is mix and left to stand at -70°C for 15 minutes. The sample spun at 14000rpm for 10 minutes/Room Temperature to pellet the DNA. The supernatant is carefully removed and the pellet washed in 70% ethanol. The DNA is re-pelleted and the supernatant removed. The pellet is dried off using a Speedi-Vac.

2.2.11 Synthesis of DNA Oligonucleotides

Oligonucleotides are synthesised at the Beatson Institute on an Applied Biosystems model 381A DNA synthesiser according to the manufacturers instructions. The machine removes 5' trityl groups, and the DNA immobilised on a column. The DNA is eluted in 29% (v/v) ammonia by passing the solution through a column approximately 20 times every 10 minutes for 1.5 hours. This solution is then sealed in a glass vial an incubated overnight at 55°C. The DNA was precipitated by the addition of ammonium acetate to 0.3M and 3 volumes of 100% ethanol. The DNA was pelleted at 7000rpm for 15 minutes in a Sorvall SS-34 rotor, resuspended in 0.2M sodium acetate, and reprecipitated in 3 volumes of ethanol. The pellet is then washed in 70% (v/v) ethanol and dissolved in 1ml of distilled water. The oligonucleotides are finally quantified by spectrophotometry.

2.2.11.1Oligonucleotides: Sequencing of p50MΔZF2zeo and p50MΔZF12zeo

5'→3' 239: aat ttg atc cag ggc aag aac cat ttt, TAD13e: aag cga att ccc tgt agg gga caa taa g, 514: aat tgg atc cca ttc tct ctc tca atg aat g, 634: aag gga tcc ccc ttc ttc atg gac cc, 804: aag ctg gat ccg aga acg gca aca tgt c, ME1: ttc atg gac ccc att tat ag, 934: ctc acc ggt gcc gcc tta cag ctc atc tc, Not1: aat tgc ggc cgc tgc tgt tca tga aga gtg aag aag, Hme1: cca gat gtc aca tga cag tgg aaa gca cta, Hme6: cta tga atg tga aaa ctg tgc cca g.

3'→5' 238: aat tgg atc caa aac ctg gtg tgt t, E1AB: cag tgt cat gtg aca tct gg, EΔ2: aat tgc ggc cgc tca tgg gtg ttt aga tag tg, 520: aag ctg gat ccg tac att gat tga gag a, EΔ7: aat tgc ggc cgc tca agt ggg tct ggc atg gct g, 724: aag ctg gat ccg tag cgc tct ttc ccc t, EΔ9: aat tgc

ggc ccgcc tca atg ttt ctt cag gtg tc, EΔ10 aat tgc ggc cgc tca tga att ttg ggc tgg ttg, EcoR1: agc tga att cat aca tgg ctt atg gac tgg at, D1: gaa tgc ggc cgc taa gca gga tgc cta ttg gtg c, EΔ4: aat tgc ggc cgc tca ctc ttt atc act ttc aag, EΔ5: aat tgc ggc cgc tca ctg gaa aaa acg gga gg.

2.2.12 Reporter Assays

2.2.12.1 Transfections

Kidney 293 cells were seeded with $1x10^6$ cells in DMEM containing 10% FCS and 2mM glutamine, twenty-four hours prior to transfection. Calcium phosphate precipitates (as described in Section 2.2.1.1) of plasmid DNA expression vectors and reporter constructs were added to 5mls of medium in each 60mm dishes of $1x10^6$ adherent 293's.
2.5μg of L8G5-CAT and 1μg of pHSV βgal were transiently transfected in the absence and presence of 25ng of lexAVP16 and with 1μg of repressor construct or PSG424. In addition, to keep the volume and concentration of DNA equal in all transfections, appropriate amounts of the bluescript vector PCH110 were added accordingly. CAT activity (quantified when less than 30% of conversion of the substrate occurs) was normalised in all samples for transfection efficiency by using the β-galactosidase activity as a control and expressed as a percentage of the reporter construct alone, which was taken as 100%.

2.2.12.2 Extract Preparation

Cells from each flask are harvested and resuspended in 1ml PBS. The samples are then spun for 10 seconds on a bench-top microfuge. The supernatant is removed and the cells re-suspended in 0.25M Tris pH7.5. The samples are then subjected to two freeze-thaw cycles (freezing at -70°C and thawing at 37°C). The extracts are then pelleted at full speed in a 4°C microfuge for 20 minutes. The supernatant is then removed and placed into a fresh eppendorf.

2.2.12.3 Cat Assays

1μl ^{14}C-Chloramphenicol, 2μl 40mM Acetyl CoA, 40μl Cell Extract and 137μl 0.25M Tris pH7.5.

The samples are then incubated at 37°C for 30 minutes. Samples are placed on ice and 500μl of ethyl acetate added to the samples to stop the reaction. The tubes are then vortexed then centrifuged for 5minutes, 13K at room temperature. The organic upper phase is then removed and transferred into fresh tubes. These samples are dried in a Speedi-Vac for 25 minutes. When the samples are dry 5μl of ethyl acetate is added to each sample. The samples are then spotted onto a silica gel TLC plate. The TLC plate is submerged in 95:5 chloroform:methanol and left for 90 minutes to run out the samples. The TLC plate is then air-dried. The plate is then exposed photographic film for 2 hours or longer. The spots are cut out and counted in a scintillation counter. Percentage conversion is calculated for each sample.

Calculation Example:

Step

1. Product/substrate x product = conversion

2. Conversion/β-galactosidase (β-gal) = X

3. X/average counts of 3X plates for 1 sample X 100 = Percentage of 100% maximum induction

2.2.12.4 β-galactosidase Assays

20μl of extract is incubated with 500μl of Solution I (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgCl_2$, 50mM β-mercaptoethanol) and 100μl of Solution II (60mM Na_2HPO_4, 40mM NaH_2PO_4, 2mg/ml ONPG). The reactions were incubated in a 37°C water bath for 10 to 90 minutes, or until the yellow colour appears. After this incubation the reactions were stopped by the addition of 250μl of 1M Na_2CO_3. The samples are read at an absorbance of 420nm in plastic 2ml cuvettes.

RESULTS AND DISCUSSION

Chapter 3: Cloning of Evi-1 Deletion Mutants into Retroviral Vectors

One principle aim of this project was to examine the biological significance of evi-1 functional domains in the transformation of mammalian cells. Since previous studies have demonstrated the requirement of DNA binding domains (ZF1 and ZF2) and repressor domain (Rp) for transcriptional repression and transformation by the evi-1 oncoprotein (Kurokawa et al., 1995 and Kilbey and Bartholomew, 1998), it was hypothesised that mutants lacking these functional domains could act as dominant negatives over full length evi-1 (FLevi-1). The recombinant p50MXneo vector (originally obtained from Dr.'s C. Stocking and W. Ostertag) containing either the entire evi-1 coding sequence, ΔZF1 mutants or ΔRp mutants were already available (obtained from C. Bartholomew). However, to fully examine these proposals several more evi-1 deletion mutant constructs had to be created.

The 8Kb p50MXneo retroviral vector is shown in Figure 5. As a retroviral vector, p50MXneo can be used as a genetic tool to integrate and express exogenous genes into a variety of animal cells, including fibroblasts and haematopoietic cells. Moreover, after transfection it allows constitutive expression of a transferred gene by strong transcription from a Myeloproliferative Sarcoma Virus Long Terminal Repeat (MPSV LTR). Furthermore, this retrovirus also contains a polylinker site for inserting donor fragments, is Neo^r and encodes both genomic and subgenomic transcripts (Figure 5). The p50MXneo construct was modified to confer resistance to the antibiotic Zeocin®to create

p50MXzeo (Figure 6). This vector was used to create new Zeo^r evi-1 deletion mutant constructs. Creating mutants in this vector not only had the advantages of the original p50MXneo vector, as described above, but also enabled the co-infection and selection of evi-1Zeo^r retroviruses in already FLevi-1Neo^r cells—a prerequisite for examining the potential dominant negative activities of evi-1 mutant proteins.

3.1 Plasmid Construction

The construction of Zeo^r vectors are discussed below. The vectors are divided into different sections according to the method of construction.

3.1.1 Plasmid Construction of p50MXzeo and p50MΔRpzeo

The p50MXneo vector was modified to p50MXzeo. Concurrently, the p50MΔRpzeo vector was created. For both constructs a 420bp zeocin fragment was derived from PCR amplification of pTracer (Invitrogen) using specific BamH1/Sal1 primer pairs (Figure 7). BamH1/Sal1 digests of the zeo fragment were inserted into pMk20 (containing the splice acceptor and polylinker sequences of p50MXneo) to create pMk20zeo. The neo cassette was then excised from both the original p50MXneo and p50MΔRpneo vectors by an EcoR1/Sal1 digest and replaced with the EcoR1/Sal1 zeo fragment from pMk20. Mini-preps of transformed colonies were examined by EcoR1/Sal1 digestion confirming that several DNAs had the correctly sized fragments (Figure 8).

3.1.2 Plasmid Construction of p50MΔZF1zeo

Evi-1 mutants lacking the first DNA binding domain or ΔZF1 mutants were inserted into the Zeo^r retroviral vectors. To create p50MΔZF1zeo a 2.5Kb ΔZF1 Not1/EcoR1 fragment from p50MΔZF1neo was inserted into the corresponding sites of the p50MXzeo construct. DNA mini-preps of transformed colonies were subjected to Not1/EcoR1 digests revealing a number of DNAs with correct fragment sizes (Figure 9).

3.1.3 Plasmid Construction of p50MΔZF2zeo and p50MΔZF12zeo

In order to examine whether evi-1 mutants lacking the second DNA domain (ΔZF2 mutants) or complete DNA binding-defective mutants (ΔZF12 mutants) could act in a dominant negative fashion over FLevi-1, two new mutant vectors were created. These mutants, p50MΔZF2zeo and p50MΔZF12zeo, were derived from PCR-amplified fragments derived from the evi-1 cDNA pBS21 (Morishita et. al., 1988) using specific primer pairs (See Figure 10 for details). Three different fragments were created (See Figure 11). Not1/BamH1 Fragment 1 (2.35Kb) and BamH1/EcoR1 fragment 2 (462bp) were used to produce ΔZF2 mutants and Not1zf12/BamH1 fragment 3 (1Kb) and fragment 2 produced the ΔZF12 mutants. The fragments were gel purified and digested with appropriate enzymes (Figure 11B). To create p50MΔZF2zeo, digested fragments 1 and 2 (Figure 11A) were simultaneously ligated into the Not1/EcoR1 site of p50MXzeo. Similarly, in order to create the ΔZF12 constructs (p50MΔZF12zeo), digested fragments 3

and 2 (Figure 11A) were simultaneously ligated into the Not1/EcoR1 site of p50MXzeo. BamH1/EcoR1/Not1 digests of DNA mini-preps indicated two ΔZF2 DNAs and four ΔZF12 DNAs with the expected fragment sizes (Figure 11C). The correct nucleotide sequences of the PCR products and cloning junctions were verified by DNA sequencing.

3.2 Expression of Evi-1 Zeo^r Deletion Mutants in Bosc23 Cells

The newly constructed vectors were tested to confirm they produced the expected proteins. Bosc23 cells were transiently transfected with p50MΔZF1zeo, p50MΔZF2zeo, p50MΔZF12zeo (Figure12A) and p50MΔRpzeo (Figure 12B) vectors and cell extracts were examined by immunoblotting using an evi-1 specific polyclonal antibody (1806). It was confirmed that the proteins encoded by these new vectors were produced at high levels in Bosc23 cells and pre-stained molecular markers indicated that all proteins migrated to their expected sizes: p50MΔZF1zeo ran at 94KDa, p50MΔZF2zeo at 137KDa, p50MΔZF12zeo at 82KDa and p50MΔRpzeo at 110Kda.

Together with previously made evi-1 retroviral vectors we now had a comprehensive collection of evi-1 deletion mutant constructs allowing thorough examination of evi-1 and the biological significance of evi-1 functional domains in the transformation of mammalian cells.

Chapter 3 Figures

Figure 5: Schematic Diagram of the p50MXneo Retroviral Vector

The diagram opposite shows a schematic of the p50MXneo vector where: MoMLV LTR is Moloney Murine Leukaemia Virus Long Terminal Repeat, MPSV LTR is Myeloproliferative Sarcoma Virus Long Terminal Repeat, Neo is the neomycin resistance gene, SA is Splice Acceptor and SD is Splice Donor.

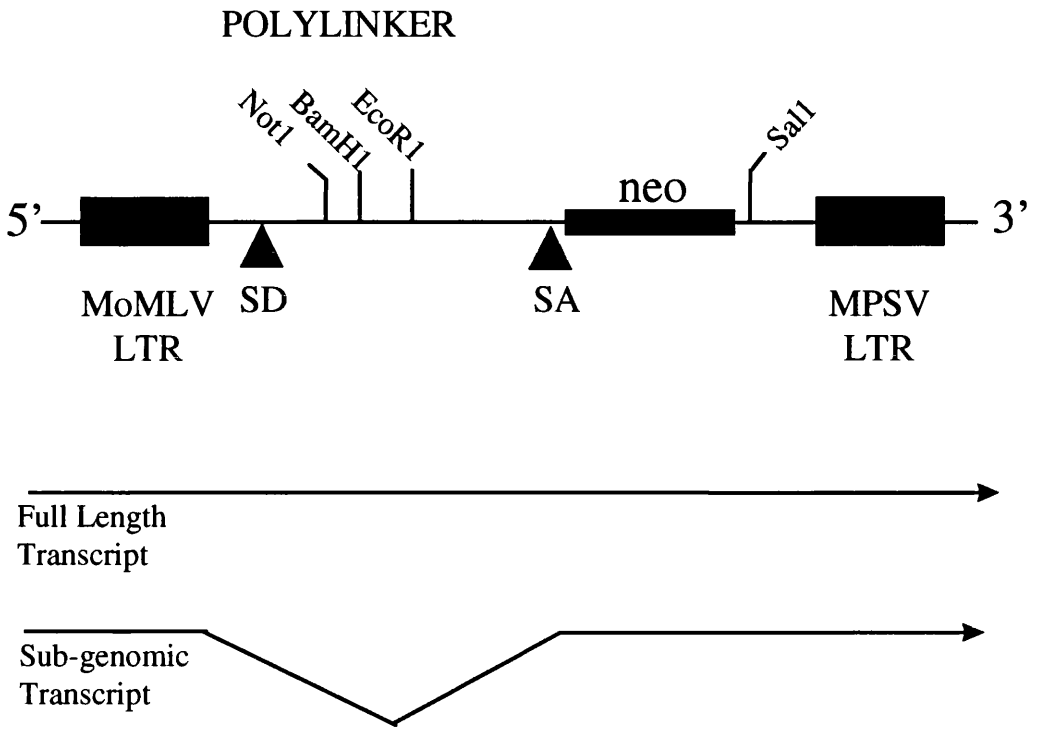

POLYLINKER
Not1
BamH1
EcoR1
Sal1
neo
5'
3'
MoMLV
LTR
SD
SA
MPSV
LTR
Full Length
Transcript
Sub-genomic
Transcript

Figure 6: Schematic Diagram of the p50MXzeo Retroviral Vector

The p50MXzeo vector was utilised to construct Evi-1 Zeo resistant (Zeo^r) deletion mutants—as described later in this chapter. The diagram opposite shows a schematic of this vector where: MoMLV LTR is Moloney Murine Leukaemia Virus Long Terminal Repeat, MPSV LTR is Myeloproliferative Sarcoma Virus Long Terminal Repeat, Zeo is the zeocin resistance gene, SA is Splice Acceptor and SD is Splice Donor.

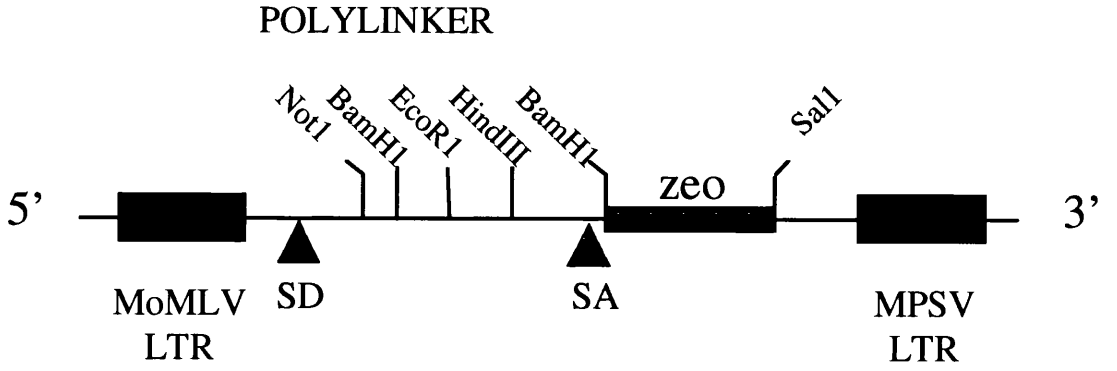
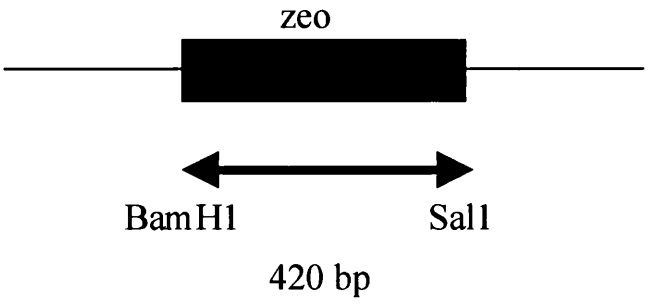

POLYLINKER
Not1
BamH1
EcoR1
HindIII
BamH1
Sal1
zeo
5'
3'
MoMLV
LTR
SD
SA
MPSV
LTR

Figure 7: Oligonucleotides Used for Creation of the 420bp Zeocin Fragment



PRIMER PAIRS

Sal1zeo	5' aat tgt cga ctc agt cct gct cct cgg 3'
BamH1zeo	5' aat tgg atc cac cat ggc caa gtt gac cag tgc 3'

Figure 8: Plasmid Construction of p50Mxzeo and p50MΔRpzeo

Arrows indicate digested vector and zeo fragments resolved on a 0.8% agarose gel with the correct fragment sizes.

MINIPREP DIGESTS

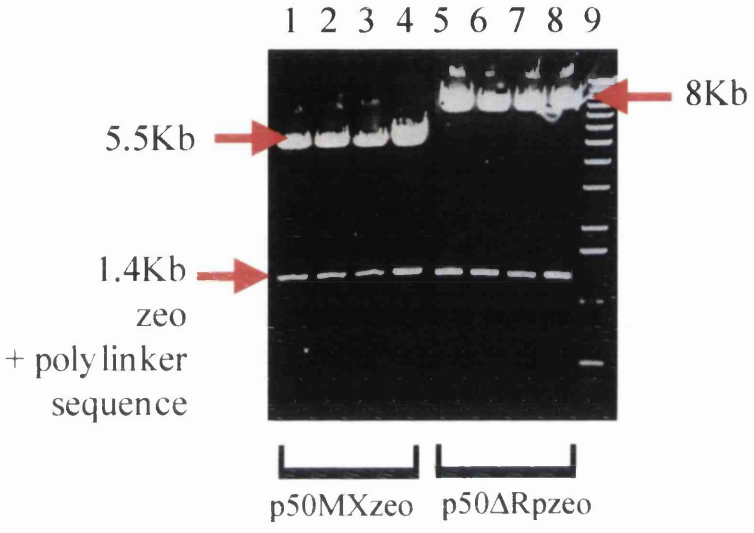


Figure 9: Plasmid Construction of p50MΔZF1zeo

Arrows indicate digests of the new p50MΔZF1zeo DNAs were resolved by agarose gel electrophoresis revealing DNAs with the correct fragment sizes (Lanes 3,5,6,8 and 9).

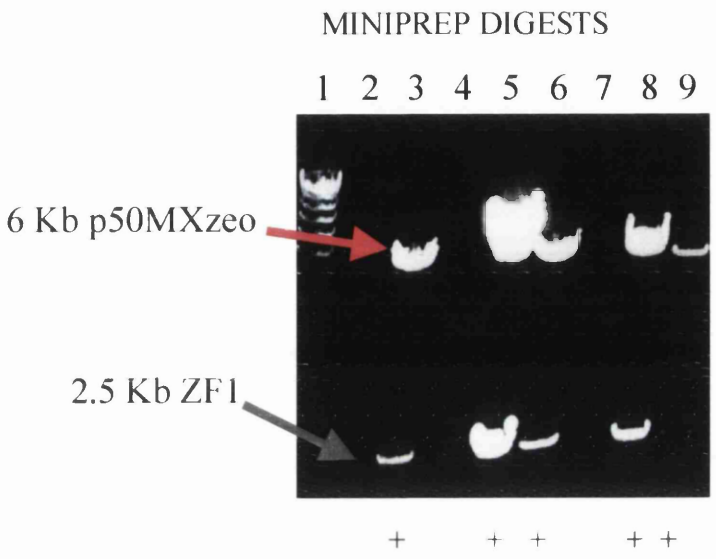

MINIPREP DIGESTS
1 2 3 4 5 6 7 8 9
6 Kb p50MXzeo
2.5 Kb ZF1
+ + + + +

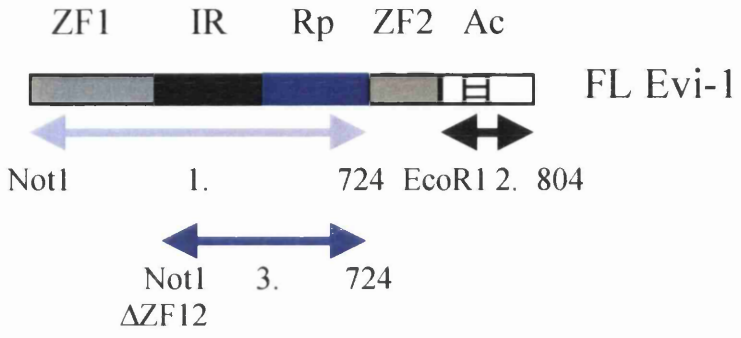
Figure 10: Oligonucleotides Used in the Cloning of p50MΔZF2zeo and p50MΔZF12zeo

A) Schematic diagram of FLEvi-1 with arrows showing the amplified fragments used to construct new deletion mutant vectors. The Evi-1 protein is shown where dark grey boxes represent zinc finger DNA binding domains (ZF1 and ZF2), the black box is the Intervening region (IR), the blue box is the repressor domain and the black/white striped box is the acidic region of the protein.

B) Oligonucleotide sequences of primer pairs used in PCR reactions.

(A+B: Pale Blue double-headed arrows represent the Not-724 amplified fragment, black double-headed arrows represent the EcoR1-804 amplified fragment and dark blue double-headed arrows represent the ΔZF12 Not1-724 amplified fragment—see text for details)

A)



B)

PRIMER PAIRS

ΔZF2

Not 1	5' aat tgc ggc cgc tgc tgt tca tga aga gtg aag agg 3'
724 (BamH1)	5' aag ctg gat ccg tag cgc tct ttc ccc t 3'
EcoR1	5' agc tga att cat aca tgg ctt atg gac tgg at 3'
804 (BamH1)	5' aag ctg gat ccg aga acg gca aca tgt c 3'

ΔZF12

Not 1 ZF12	5' aat tcg ggc cgc tgc tgt tca tgg agg gca aga acc att 3'
724 (BamH1)	5' aag ctg gat ccg tag cgc tct ttc ccc t 3'
EcoR1	5' agc tga att cat aca tgg ctt atg gac tgg at 3'
804 (BamH1)	5' aag ctg gat ccg aga acg gca aca tgt c 3'

Figure 11: Plasmid Construction of p50MΔZF2zeo and p50MΔ ZF12zeo

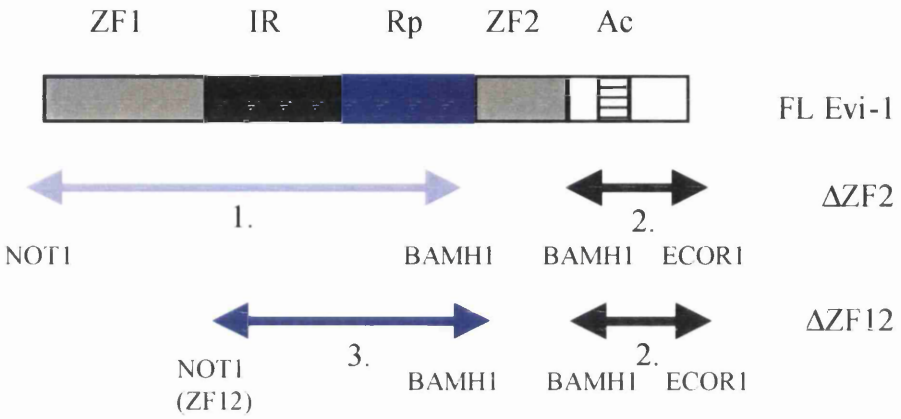
A) Schematic showing fragments amplified for the construction of p50MΔZF2zeo and p50MΔZF12zeo mutants. (See Figure 10 for key to Evi-1 functional domains and fragment colour coded arrows).

B) Arrows indicate the p50MΔZF2zeo and p50MΔZF12zeo Evi-1fragments PCR-amplified from pBS21 (original plasmid vector containing full length murine evi-1 cDNA). The fragments are gel purified and digested and resolved on a 0.8% agarose gel. (Fragment "1" = Lane 2, Fragment "2" = Lanes 3 and 5 and Fragment "3" = Lane 4).

C) Arrows indicate new p50MΔZF2zeo and P50MΔZF12zeo DNAs, which have been digested and checked on a 0.8% agarose gel. The integrity of the new DNA was checked by sequencing.

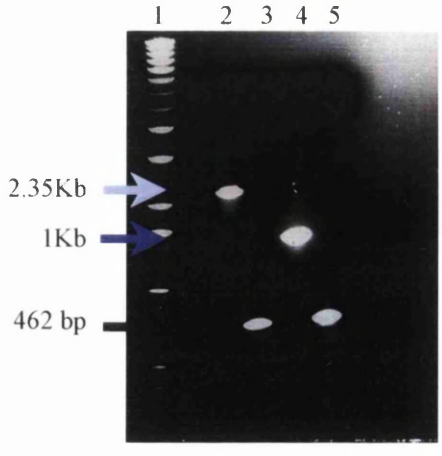
(A,B + C: Fragment "1" = Not1-724 fragment, Fragment "2" = BamH1-EcoR1 fragment and Fragment "3" = ΔZF12Not1-724 fragment—see figure 10 for arrow colour-coding)

A) PCR



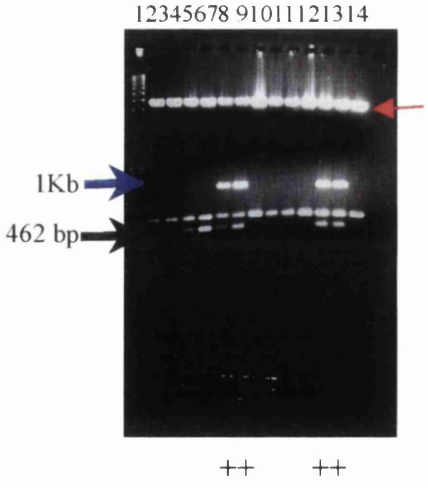
LIGATE/TRANSFORM

B) DIGEST+PURIFY



C) MINIPREP DIGESTS

ΔZF12



ΔZF2

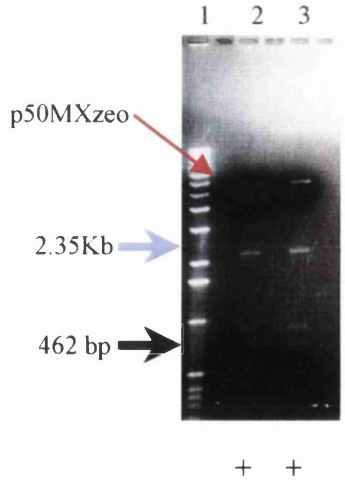
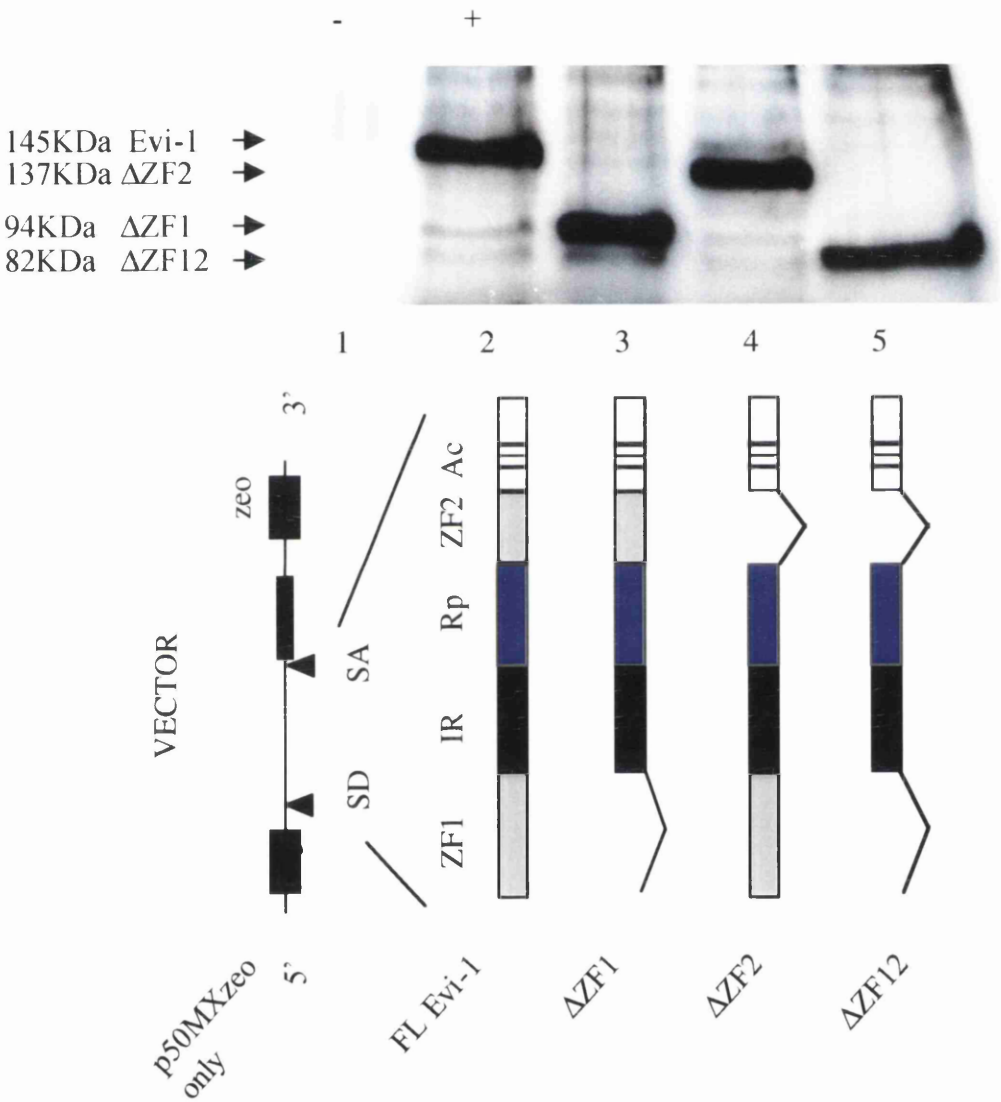


Figure 12: Western Blot Analysis of Bosc23 Cells Transfected with New Mutant Zeo^r Constructs

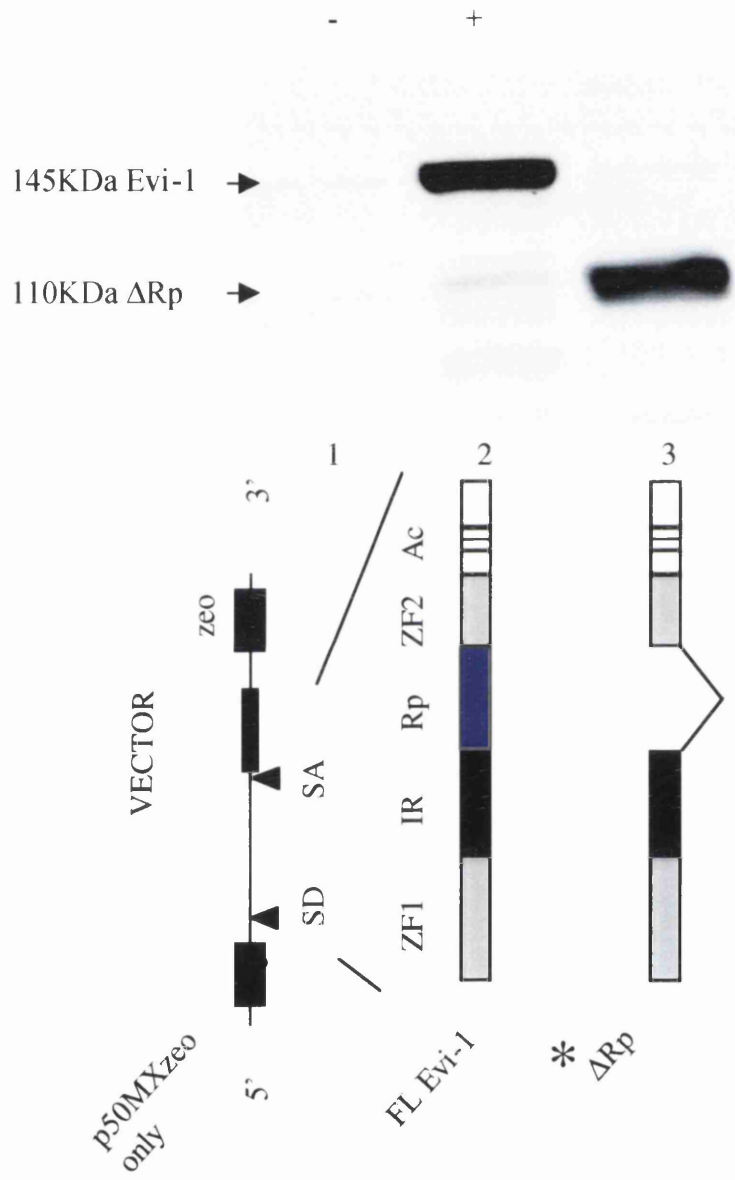
A) Whole cell extracts of Bosc23 cells transfected with: Lane 1, p50MXzeo - Negative Control. Lane 2, p50MFLneo -Positive Control. Lane 3, p50MΔZF1zeo. Lane 4, p50MΔZF2zeo. Lane 5, p50MΔZF12zeo. Proteins were fractioned on a 10% SDS-polyacrylamide gel and analysed by Western Blot with the anti-Evi-1 monoclonal antibody (1806). Arrows indicate the location of FLEvi-1, ΔZF1, ΔZF2 and ΔZF12 mutant proteins.

A)


-
+
145KDa Evi-1
137KDa ΔZF2
94KDa ΔZF1
82KDa ΔZF12
1
2
3
4
5
VECTOR
zeo
3'
5'
SD
SA
IR
Rp
ZF2
Ac
ZF1
p50MXzeo only
FL Evi-1
ΔZF1
ΔZF2
ΔZF12

B) Whole cell extracts of Bosc23 cells transfected with: Lane1, p50MXzeo -Negative Control. Lane 2 p50MFLneo -Positive Control. Lane 3, p50MΔRpzeo. Proteins were fractioned on a 7.5% SDS-polyacrylamide gel and analysed by Western Blot with the anti-Evi-1 monoclonal antibody (1806). Arrows indicate the location of FLEvi-1 and ΔRp mutant proteins.

B)


-
+
145KDa Evi-1
110KDa ΔRp
1
2
3
3'
zeo
VECTOR
SA
SD
5'
p50MXzeo only
Ac
ZF2
Rp
IR
ZF1
FL Evi-1
*
ΔRp

Chapter 4: Investigating Evi-1 Deletion Mutants as Potential Dominant Negative Inhibitors of Evi-1-mediated Transformation of Rat1 cells

Previous studies have demonstrated that the transformation of Rat1 cells by constitutive expression of Full Length evi-1 (FLevi-1) depends on certain functional domains of the protein (Kurokawa et al., 1995, Bartholomew et al., 1997 and Kilbey and Bartholomew, 1998). A Rat1 model system was adapted to examine whether the non-transforming mutants of evi-1, characterised as a result of this earlier work, would act in a dominant negative fashion over FLevi-1 proteins in Rat1 cells constitutively expressing Full Length evi-1 or Rat1FL cells (Kilbey et al., 1999). As a consequence a reversion of the transformation in these cells was expected. Ultimately, it was envisaged that such mutant inhibitors would be used as reagents to probe the function of EVI-1 in leukaemia.

The model system used in these studies was based on the Rat1 Transformation Assay. Figure 13 gives a brief overview of the original assay:

4.1 Evi-1 Transformation is Dependent on Functional Domains of the Protein

To examine which domains of evi-1 are essential for the transformation of Rat1 cells the transforming activities of several constructs either expressing naturally occurring evi-1 isoforms or evi-1 deletion mutants were examined. Rat1 cells expressing either FLevi-1 (p50MFLevi-1neo), the Δ324 isoform (p50MΔ324neo) or mutants lacking the repressor

repressor domain Rp (p50MΔRpneo), the first DNA binding domain ZF1 (p50MΔ ZF1neo) or the intervening region IR (p50MΔIRneo) were obtained from Dr. A. Kilbey. These cell lines have been described previously (Kilbey and Bartholomew, 1998). In this study, populations of Rat1 cells infected with mutants lacking the second DNA binding domain ZF2 (p50MΔZF2zeo) or both DNA binding domains (p50MΔZF12zeo) were isolated as described in Chapter 2 Section 2.2.4. Rat1 populations expressing either p50MXneo or p50MXzeo vectors were used as controls. Figure 14A shows Western blot analysis with an anti-evi-1 specific antibody of the respective evi-1 mutant proteins expressed in these populations. This analysis revealed the expected size proteins. Results from soft agar studies show that mutants lacking major features of the evi-1 protein (namely ZF1,ZF2 and the Rp domain) are unable to exhibit the transforming activity displayed by FL proteins in Rat1 fibroblasts (Figure 14B). Mutants where the intervening region (IR) was deleted remained as transforming as FLevi-1 controls demonstrating that this region is not necessary for evi-1-mediated transformation.

4.2 Dominant Negative Studies

4.2.1 Revising the Original Rat1 Transformation Assay

The original transformation assay was modified so that potential dominant negative effects of mutant evi-1 proteins could be investigated (Figure 15). Bosc23 cells are transfected with mutant evi-1 retroviral DNA constructs carrying the zeocin resistance gene. (For construction of Zeo^r plasmids see Chapter 3). Viral supernatant is harvested and then used to infect Rat1FL cells. Cells are selected in both zeocin® and G418, then

populations and single cell clones are isolated. Western blot analysis confirms the translation of both FL and the expected sized mutant proteins in each isolated population or clone. These cell lines expressing high levels of both FLevi-1 and a deletion mutant of evi-1 are re-introduced into soft agar assays to assess their phenotype.

4.2.2 Isolation of Cell Lines co-expressing FLevi-1 and Non Transforming Evi-1 Deletion Mutants

Initially, four new cell types were established by the method described in 4.2.1. Four zeocin resistant vectors (also described in Chapter 3), Zeo^r vector only (p50MXzeo), evi-1 mutants lacking the first DNA binding domain (p50MΔZF1zeo), evi-1 mutants lacking the second DNA binding domain (p50MΔZF2zeo), evi-1 mutants lacking the repressor domain (p50MΔRpzeo) and evi-1 mutants lacking both DNA binding domains (p50MΔ ZF12zeo) were separately introduced into Rat1FL cells. Populations of Rat1 cells expressing these mutants had no effect on evi-1-mediated transformation of Rat1 cells. Therefore, three or more independent clones were isolated for each new cell line. After two weeks selection in both zeocin® and G418 individual resistant clones were grown up and the expression of both evi-1 and evi-1 deletion mutants were analysed by Western blot analysis with an anti-evi-1 specific anti-body.

In Figure 16, Lanes 3 and 4 show the Western Blot analysis of two clones expressing FLevi-1 and p50MXzeo vector (Rat1FL/Xzeo cells). These clones provided positive control cell lines for the dominant negative studies. It was hypothesised that these cell

lines would have identical transforming properties to Rat1FL cells. Western Blot analysis of three clones expressing both FLevi-1 and ΔRp mutants (Rat1FL/ΔRp cells) (Figure 17, Lanes 3-4) revealed that the levels of Rp tend to slightly exceed that of FLevi-1. The expression of FL evi-1 and ΔZF1 mutants in Rat1 cells (Rat1FL/ΔZF1 cells) co-expressing these two proteins appears to be similar in all three clones examined (Figure 18, Lanes 3-5). Only one clone co-expressing FLevi-1 and ΔZF2 mutants (Rat1FL/ΔZF2 cells) with detectable amounts of ΔZF2 protein was isolated out of ten G418/zeo resistant clones. Interestingly, Western Blot analysis of this clone reveals that expression of FLevi-1 is dramatically decreased in this clone with a clear favouring for very high levels of the ΔZF2 mutant (Figure 19). Finally, three clones with detectable levels of both FLevi-1 and ΔZF12 mutants are shown in Figure 20 (Lanes 3-5). It was noted that expression levels of ΔZF12 proteins were favoured over FLevi-1 in these cell lines co-expressing both of these proteins.

4.2.3 ΔZF1 Mutants, ΔRp Mutants and ΔZF12 Mutants of Evi-1 act as Dominant Negatives over FLevi-1

The phenotype of cell lines co-expressing FLevi-1 and either ΔZF1 mutants, ΔZF2 mutants or ΔRp mutants of evi-1 were examined by assessing their growth in soft agar.

The histogram in Figure 21 shows the results of triplicate plates examined for each cell line from a typical experiment. These results demonstrate that the expression of mutants lacking either the ZF1 domain or the Rp region of evi-1 can revert the transformed

phenotype of Rat1FL cells. A significant reduction in colony number was observed compared to Rat1FL/XZeo controls. Interestingly, ΔZF12 mutants which completely lack DNA binding domains dramatically reduce evi-1-mediated transformation in Rat1 cells in an identical manner.

4.2.4 Co-operative transformation of Rat1 cells by FLevi-1 and ΔZF2 Mutants

The constitutive activity of ΔZF2 mutants in Rat1FL cells appears to stimulate their transforming activity. The significant synergistic effect observed in these cells expressing FLevi-1 and ΔZF2 mutants was coupled with an altered morphology of resulting clones. The photograph C in Figure 22 shows the promotion of the transformed morphology of these clones. The colonies were enormous with irregular edges and most clones were >0.8cm in diameter. Only one independent clone was used in this analysis due to the lack of detectable expression of ΔZF2 protein in other $G418^r/Zeo^r$ Rat1FL/ΔZF2 clones (Figure 19B). Therefore isolation of other such clones are required to confirm this result.

4.3 Discussion

4.3.1 ΔZF1 mutants, ΔRp mutants and ΔZF12 mutants act in a Dominant Negative Fashion over FLevi-1

Previous studies have recognised that the biological properties attributed to evi-1 are dependent on the ZF1, ZF2 and Rp functional domains of the protein (Bartholomew et al., 1997, Kurokawa et al., 1995 and Kilbey and Bartholomew, 1998). This work was

reproduced and verified in these studies. It was postulated that mutants lacking these important biological domains of evi-1 could be potential candidates for dominant negative inhibition of FLevi-1 oncogenic activity in Rat1 cells. Here, three evi-1 deletion mutants have been created and characterised that exhibit dominant negative action over FLevi-1 resulting in a significant reduction in evi-1-mediated transformation of Rat1 fibroblasts: namely ΔRp mutants, ΔZF1 mutants and ΔZF12 mutants all completely abrogated evi-1-mediated macroscopic colony growth in soft agar.

4.3.2 Possible Mechanisms of Dominant Negative Action

If the molecular mechanism/s of these inhibitors can be defined, the identification of the pathways and intermediaries involved in EVI-1 transforming activity will be forthcoming. The results suggest that distinct pathways may be used by dominant negative mutants to render Rat1FLevi-1 cells with the same reverted phenotype.

4.3.2.1 "Blocking" Mechanism

With partially and fully intact DNA binding domains respectively, the ΔZF1 and ΔRp mutants could inhibit transformation through a "blocking" mechanism where mutants compete with FLevi-1 for evi-1 DNA binding sites (Figure 23B). To address this possibility ZF1 and ZF2 domains only should be expressed in RatFL cells. As before, the ability of these domains to block evi-1 transforming activity can be assessed in the Rat1 transformation assay.

4.3.2.2 "Squelching" Mechanism

The ΔZF12 mutants, completely defective in DNA binding, also impair evi-1-mediated transformation. Therefore, these studies suggest that evi-1 protein-protein interactions mediate the dominant negative activity of these mutants. The DNA binding-defective mutant ZF12 could utilise so-called "squelching" or sequestration mechanisms to achieve this biological action (Figure 23C). "Squelching" mechanisms have been described as the mechanisms whereby an inhibitor interacts with endogenous factors that are involved in the regulation of transcription but not bound to DNA. In past studies when "squelching" of transcription factor function has been demonstrated, very high levels of expression are required. Moreover, this mechanism appears to be much more difficult to achieve when compared to "quenching" or "blocking" mechanisms (Gill and Ptashne, 1988, Berger et. al., 1990 and Ptashne and Gann, 1990). Interestingly, if we recall Western blot analysis (Figure 20) of individual Rat1 FLevi-1/ΔZF12 clones, the relative expression of these proteins is such that the ΔZF12 expression levels are higher than FLevi-1 protein levels. This favouring of the dominant negative protein was not such an obvious feature in Rat1 FL/ΔZF1 or Rat1FL/ΔRp clones. Indeed there are several examples of an apparent "threshold" level of the FL protein being crucial for certain biological activities of evi-1. The choice of repressor pathway appears to be determined by levels of the FL protein. The literature states that optimal evi-1 repressor activity requires binding to DNA through ZF1 (Bartholomew et al., 1997). In the same studies DNA binding independent repression was observed at higher intracellular protein concentrations of evi-1. Others have shown that high levels of evi-1 are critical for blocking the responsiveness of haematopoietic cells to granulocyte-colony stimulating factor (G-CSF) (Khanna-Gupta et

al., 1996). Perhaps then it is the levels of dominant negative protein that determines their mode of inhibitory action.

So which domain of the ΔZF12 mutant could "squelch" proteins involved in the evi-1 pathway? The most biologically important domain remaining in the ΔZF12 mutants and relevant to transformation is the repressor region. Therefore, it is reasonable to assume that the high levels of ΔZF12 protein observed in FLevi-1/ΔZF12 Rat1 cells could inhibit evi-1 transformation through a "squelching" mechanism involving the sequestration of endogenous FLevi-1 regulatory proteins through the repressor domain.

Interestingly, there are a considerable number of reports emerging that highlight the importance of transcriptional repressor activity of oncoproteins in the molecular pathogenesis of myeloid leukaemias. Specifically, the aberrant formation of stable corepressor complexes is paramount to the leukaemogenic potential of certain fusion oncoproteins. Examples of this include the acute myeloid leukaemia 1/eight-twenty one (AML1/ETO) and promyelocytic leukaemia/retinoic acid receptor (PML/RAR) transforming fusion proteins of Acute Myeloid Leukaemia (AML) and Acute Promyelocytic Leukaemia (APL)—both of which function as repressors by the aberrant recruitment of a nuclear receptor corepressor (Co-R)/Sin3-histone deactetylase (HDAC) complex (Lutterbach et al., 1998 and Grignani et al., 1998 respectively). It has been suggested that consequent alterations in chromatin structure and other effects on transcriptional regulation could be responsible for the differentiation block associated with the expression of these oncogenes in myeloid leukaemogenesis. Moreover, involvement of these aberrant corepressor complexes in two genetically distinct forms of

myeloid leukaemia strengthens the importance of this repression pathway in myeloid cell differentiation.

Are corepressor complexes also critical for the biological activities of EVI-1? This is tempting to speculate because the AML1 and PML oncogenic proteins exhibit many parallels with EVI-1, with the major examples being: (1) EVI-1 encodes an oncoprotein (Kurokawa et al., 1995) ; (2) EVI-1 is also a repressor of transcription (Bartholomew, 1997) ; (3) Evi-1 also forms a oncogenic fusion protein—the AML1/EVI-1 fusion is found in cases of t(3;21) chronic myeloid leukaemia (CML) (Mitani et al,, 1994) and (4) Constitutive evi-1 expression also blocks the production of several lineage-specific haematopoietic progenitors (Morishita et al., 1992, Kreider et al., 1993).

4.3.2.3 "Quenching" by Heterodimerisation

Finally, the possibility that EVI-1 can bind to itself—perhaps through the acidic domain —cannot be ruled out. Acidic regions are typical dimerisation domains of proteins (Chen and Bieker, 1996 and Geiman et al., 2000) and the acidic region of evi-1 is still intact in all the dominant mutants tested. One possible scenario would be that all of the evi-1 deletion mutants could heterodimerise with evi-1 interfering with FLevi-1 function. These heterodimers could either stoichiometrically inhibit evi-1 sequence-specific binding or "quench" the activity of FLevi-1 by binding to evi-1 binding sites and subsequently inhibiting FLevi-1 transformation (Figure 23D). Performing gel-shift or co-immunopreciptation experiments with FLevi-1 and mutant evi-1 proteins could identify the formation of dimeric complexes of evi-1 and thus determine its self-association.

4.3.3 Co-operative transformation of Rat1 cells by FLevi-1 and Mutants Lacking the Second DNA Binding Domain (ΔZF2 Mutants)

Surprisingly, evi-1 proteins lacking the second DNA binding domain (ΔZF2 mutants) do not act as dominant negative mutants. Interestingly, unlike the Rat1FL/ΔZF1, Rat1FL/ΔRp and Rat1FL/ΔZF12 cells, the majority of Rat1FL/ΔZF2 clones isolated post-selection did not survive in tissue culture. Furthermore, Rat1 cells seemed adverse to allowing co-expression of FLevi-1 and the ΔZF2 mutant—as illustrated in Figure 19B. Therefore, finding a clone co-expressing FLevi-1 and ΔZF2 mutants with detectable Δ ZF2 protein expression levels proved difficult, and consequently only one clone of eight examined, was isolated. Western blot analysis revealed very high protein levels of ΔZF2 and almost undetectable FLevi-1 levels in this clone. Furthermore, introduction of this clone into soft agar assays revealed a synergistic transforming effect enhancing transformation compared to Rat1FL/Rat1FLXzeo controls. Interestingly, these clones displayed a more severe transformed phenotype than FLevi-1 controls (Figure 22C). The colonies were generally double the size of controls, very dense and with invasive irregular edges of growing cells. These studies are in conflict with previous findings that populations of Rat1 cells expressing ΔZF2 mutants are not transforming (as the ΔZF1, ΔZF12 and ΔRp mutants)—suggesting the effect seen here may simply be clonal. Furthermore, on the basis of one solitary clone it is impossible to consider the significance of this result.

4.4 Summary

Three potent dominant negative inhibitors of evi-1 oncogenic activity have been identified in these studies. From the evidence described in this chapter, it is proposed that this inhibitory activity of dominant negative mutants can be mediated through either direct DNA binding or by interference with regulatory proteins necessary for evi-1-mediated transformation. It appears that these mutants will provide the useful genetic tools necessary for unravelling the complex cellular processes involved in the functioning of the *EVI-1* oncogene in mammalian cells.

Chapter 4 Figures

Figure 13: The Rat1 Transformation Assay—Methodology

Bosc23 cells are transiently with evi-1 retroviral DNA constructs that carry neomycin resistance. Viral supernatant is harvested and then used to infect Rat1 cells. These cells, infected with retrovirus, are selected in G418 and populations or single cell clones are isolated. Expression of the evi-1 proteins is examined by Western blot analysis and the transformed phenotype of cells determined by growth in soft agar. The colonies scored are ≥0.1cm in diameter following a 3-5 week incubation.

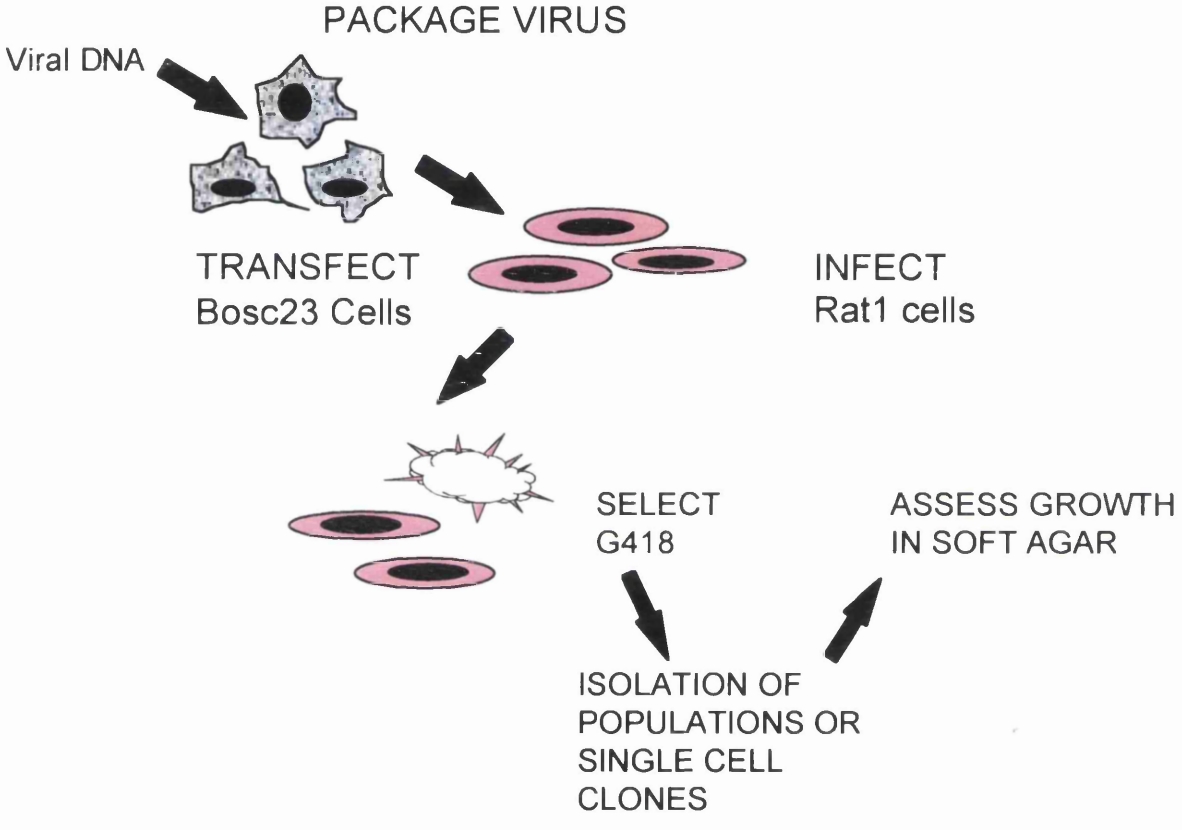
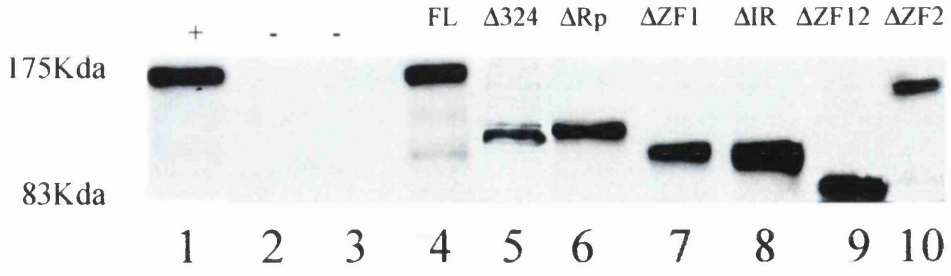

PACKAGE VIRUS
Viral DNA
TRANSFECT
Bosc23 Cells
INFECT
Rat1 cells
SELECT
G418
ASSESS GROWTH
IN SOFT AGAR
ISOLATION OF
POPULATIONS OR
SINGLE CELL
CLONES

Figure 14: Transformation of Rat1 Fibroblasts by Evi-1 Requires ZF1, ZF2 and Rp Domains

A) Western blot analysis of whole cell extracts derived from Rat1 cells constitutively expressing FLevi-1 and evi-1 deletion mutants. Proteins from Rat1 cells transfected with p50FLevi-1neo and evi-1 deletion mutants - as illustrated opposite - were fractioned on a 7.5% SDS-polyacrylamide gel and subjected to Western blot analysis with a specific anti-evi-1 antibody (1806). Lane 1 (+) is a positive control of Rat1 cells expressing p50MFLevi-1neo, Lane 2 (-) is a negative control of Rat1 cells only, Lane 3 (-) is a negative control Rat1MX cells, Lane 4 (-) is a negative control Rat1MXZeo cells, Lane 5 is Rat1FL cells, Lane 6 is Rat1 cells expressing p50MΔ324neo, Lane 7 is Rat1 cells expressing p50MΔRpneo, Lane 8 Rat1 cells expressing p50MΔZF1neo, Lane 9 is Rat1 cells expressing p50MIRneo, Lane 10 is Rat1 cells expressing p50MΔZF12zeo and Lane 11 is Rat1 cells expressing p50MΔZF2zeo.

B) The histogram opposite shows the results from transformation assays using these clones. These experiments were carried out at least twice and those shown are in triplicate for each cell type. The DNA binding domains (grey boxes), Intervening Region (IR-blue boxes), Acidic Domain (Ac-black/white striped box), Repressor Domain (Rp-dark blue box) of evi-1 and the Long Terminal Repeats (LTR-blue/white striped box) and neomycin/zeocin resistance gene (neo/zeo-dark grey boxes) of the retroviral vectors are indicated. Standard deviations ± of the mean are shown.

A)


+
-
-
FL
Δ324
ΔRp
ΔZF1
ΔIR
ΔZF12
ΔZF2
175Kda
83Kda
1 2 3 4 5 6 7 8 9 10

B)

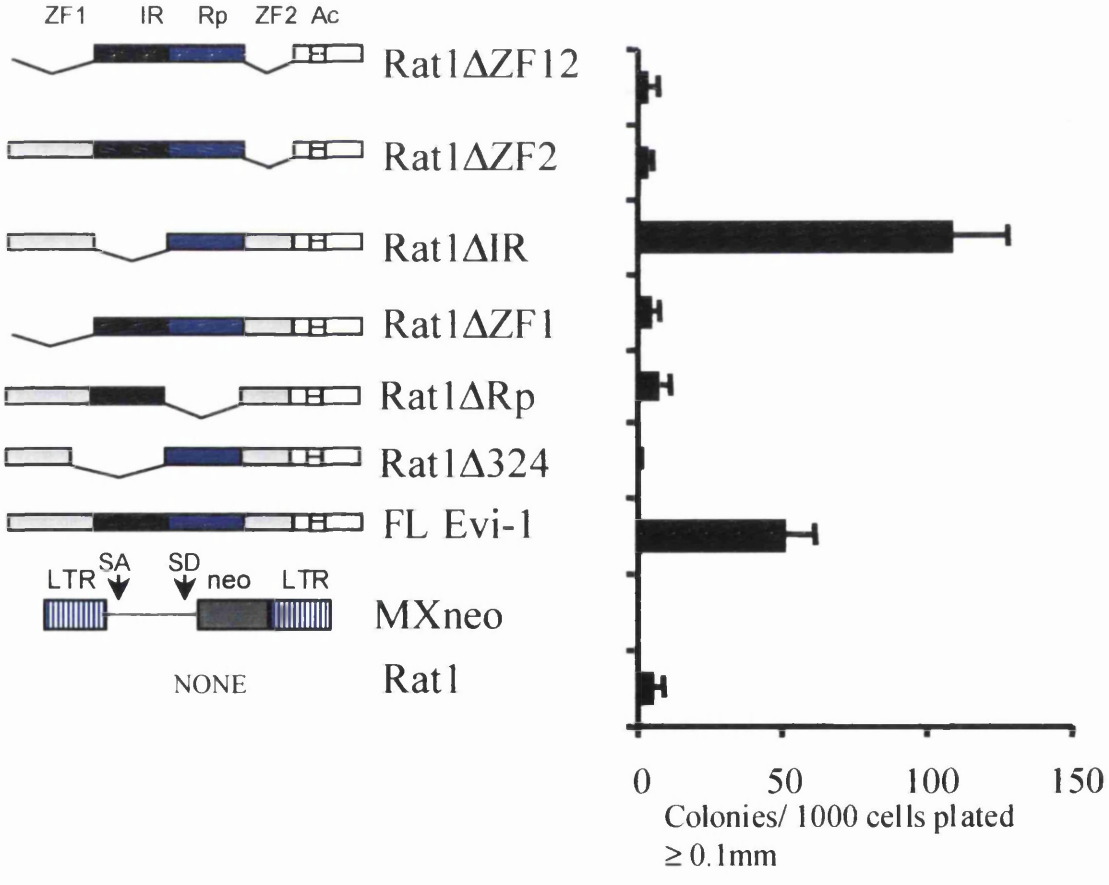

ZF1
IR
Rp
ZF2
Ac
Rat1ΔZF12
Rat1ΔZF2
Rat1ΔIR
Rat1ΔZF1
Rat1ΔRp
Rat1Δ324
FL Evi-1
LTR
SA
SD
neo
LTR
MXneo
NONE
Rat1
0
50
100
150
Colonies/ 1000 cells plated
≥ 0.1mm

Figure 15: The Revised Transformation Assay

See text for details.

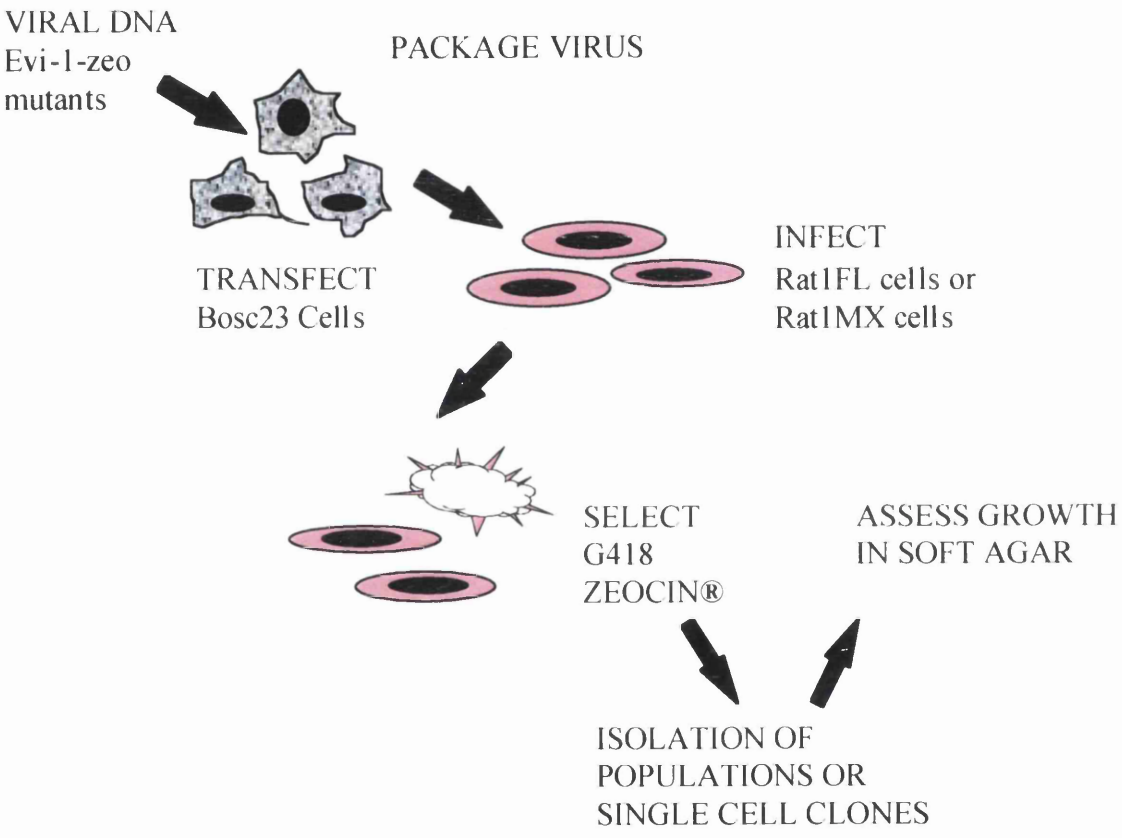

VIRAL DNA
Evi-1-zeo
mutants
PACKAGE VIRUS
TRANSFECT
Bosc23 Cells
INFECT
Rat1FL cells or
Rat1MX cells
SELECT
G418
ZEOCIN®
ASSESS GROWTH
IN SOFT AGAR
ISOLATION OF
POPULATIONS OR
SINGLE CELL CLONES

Figure 16: Western Blot Analysis of Rat1FL/Xzeo Cells

Proteins from whole cell extracts from Rat1 cells were fractioned on a 7.5% SDS-polyacrylamide gel and subjected to Western Blot analysis with a specific anti-evi-1 polyclonal antibody (1806). Lane 1 is a Positive control of Rat1FL cells. Lane 2 is a Negative control of Rat 1 cells containing the p50MXneo vector alone. Lanes 3 and 4 are two independent Rat1FL clones containing p50MXzeo. These clones provide essential control cell lines for the dominant negative studies. The arrow indicates the location of FLevi-1proteins.

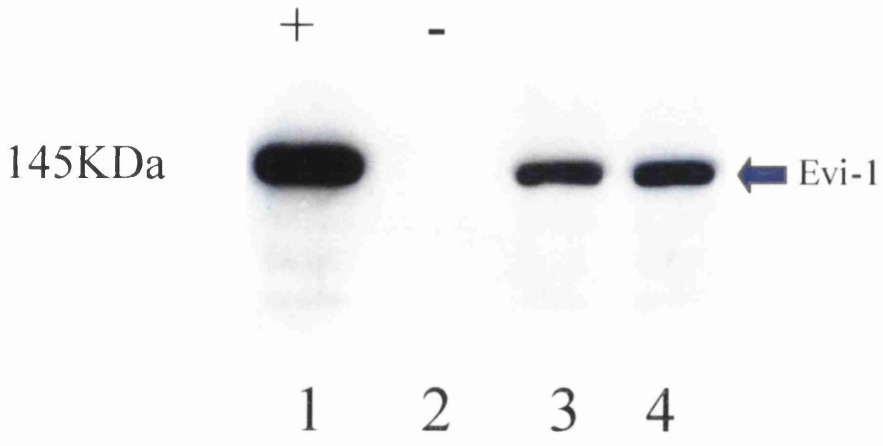

+
-
145KDa
Evi-1
1
2
3
4

Figure 17: Western Blot Analysis of Rat1FL/ΔRp Clones

Proteins from whole cell extracts were fractioned on a 7.5% SDS-polyacrylamide gel and subjected to Western blot analysis using a specific anti-evi-1 polyclonal antibody (1806). Lane 1 (+) is a Positive control of Rat1FL cells. Lane 2 (-) is a Negative control Rat1MX cells. Lanes 3 and 4 are independent Rat 1FL/ΔRp clones. Arrows indicate the location of FLevi-1 and ΔRp proteins.

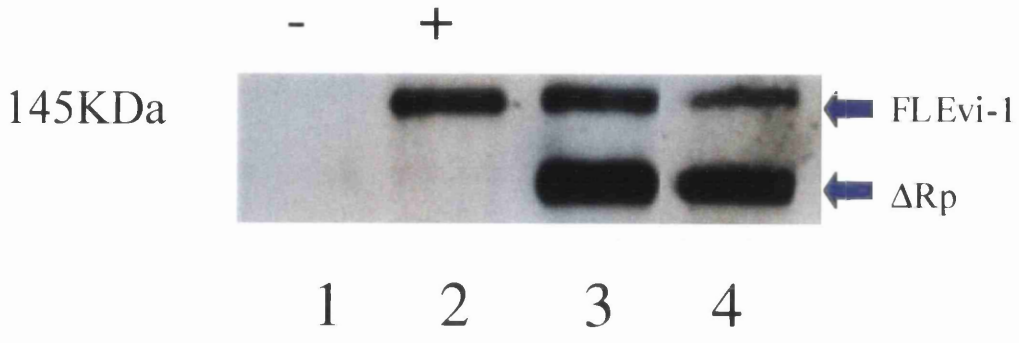

-
+
145KDa
FLEvi-1
ΔRp
1
2
3
4

Figure 18: Western Blot Analysis of Rat1FL/ΔZF1 Clones

Proteins from whole cell extracts were fractioned on a 10% SDS-polyacrylamide gel and subjected to Western blot analysis using a specific anti-evi-1 polyclonal antibody (1806). Lane 1 (-) is a Negative control of Rat1 cells transfected with p50MXneo vector only. Lane 2 is a Positive control of Rat1FL cells. Lanes 3 to 5 are three independent clones expressing p50MFLevi-1neo and p50MΔZF1zeo. Arrows indicate the location of FLevi-1and ΔZF1 proteins.

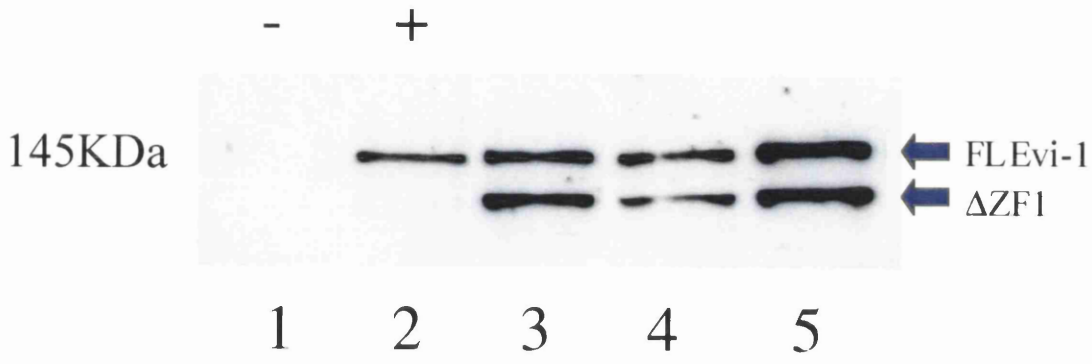
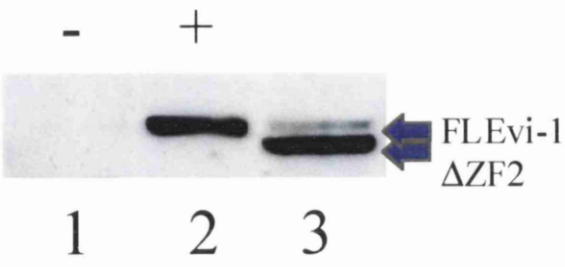

-
+
145KDa
FLEvi-1
ΔZF1
1
2
3
4
5

Figure 19: Western Blot Analysis of Rat1FL/ΔZF2 Clones

A) Proteins from whole cell extracts were fractioned on a 7.5% SDS-polyacrylamide gel subjected to Western blot analysis using a specific anti-evi-1 polyclonal antibody (1806). Lane 1 (-) is a Negative control of Rat1MX cells. Lane 2 is a Positive control of Rat1FL cells. Lane 3 is one clone of Rat1FL/ΔZF2 cells. Arrows indicate the location of FLevi-1and ΔZF2 proteins.

B) Western Blot analysis of several ΔZF2 clones. Lane 1 (-) is a Negative control of Rat1MX cells. Lane 2 is a Positive control of Rat1FL cells. Lane 3-8 are clones of Rat1FL/ΔZF2 cells. Arrows indicate the location of FLevi-1and ΔZF2 proteins.

A)



B)

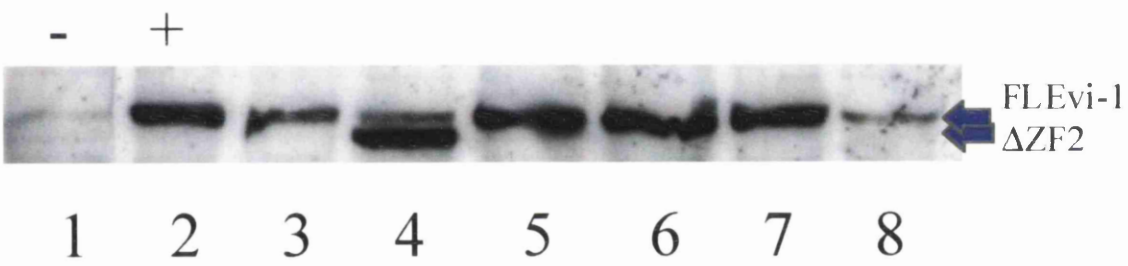


Figure 20: Western Blot Analysis of Rat1/ΔZF12 Clones

Proteins were fractioned on a 7.5% SDS-polyacrylamide gel subjected to Western blot analysis using a specific anti-evi-1 polyclonal antibody (1806). Lane 1 (-) is a Negative control of Rat1 cells transfected p50MXneo vector only. Lane 2 is a Positive control of Rat1 cells transfected with p50FLevi-1neo. Lanes 3 to 5 are three independent clones expressing p50MFLevi-1neo and p50MΔZF12zeo. Arrows indicate the location of the FLevi-1 and ΔZF12 proteins.

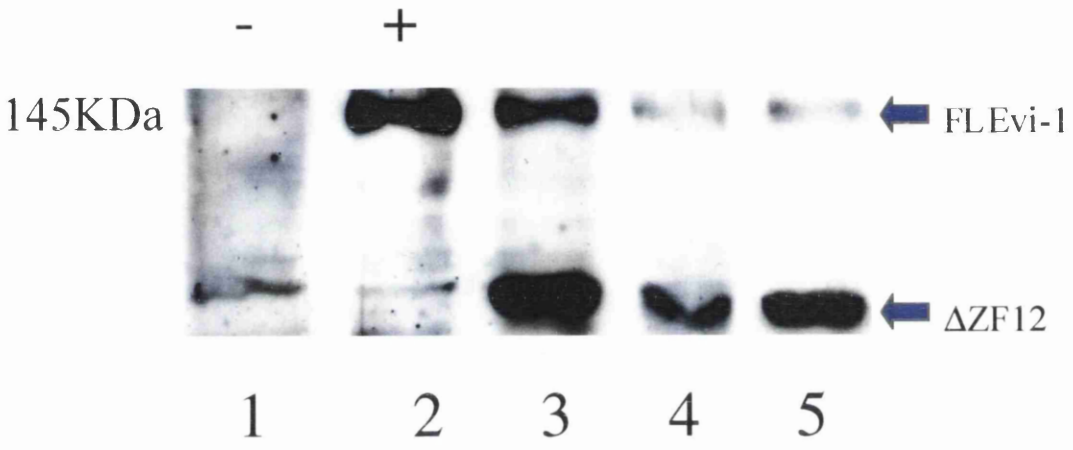

-
+
145KDa
FLEvi-1
ΔZF12
1
2
3
4
5

Figure 21: Evi-1 ΔRp, ΔZF1 and ΔZF12 Deletion Mutants Revert the Transformed Phenotype of Evi-1-Transformed Rat1 Fibroblasts

The histogram opposite illustrates the results from one representative clone from each of these cell lines. Results shown are the mean ± Standard Error of the triplicate plates examined for each clone. Two or more clones were examined in triplicate for each deletion mutant tested . For colour coding see Figure 14B.

Rat1Cells expressing:

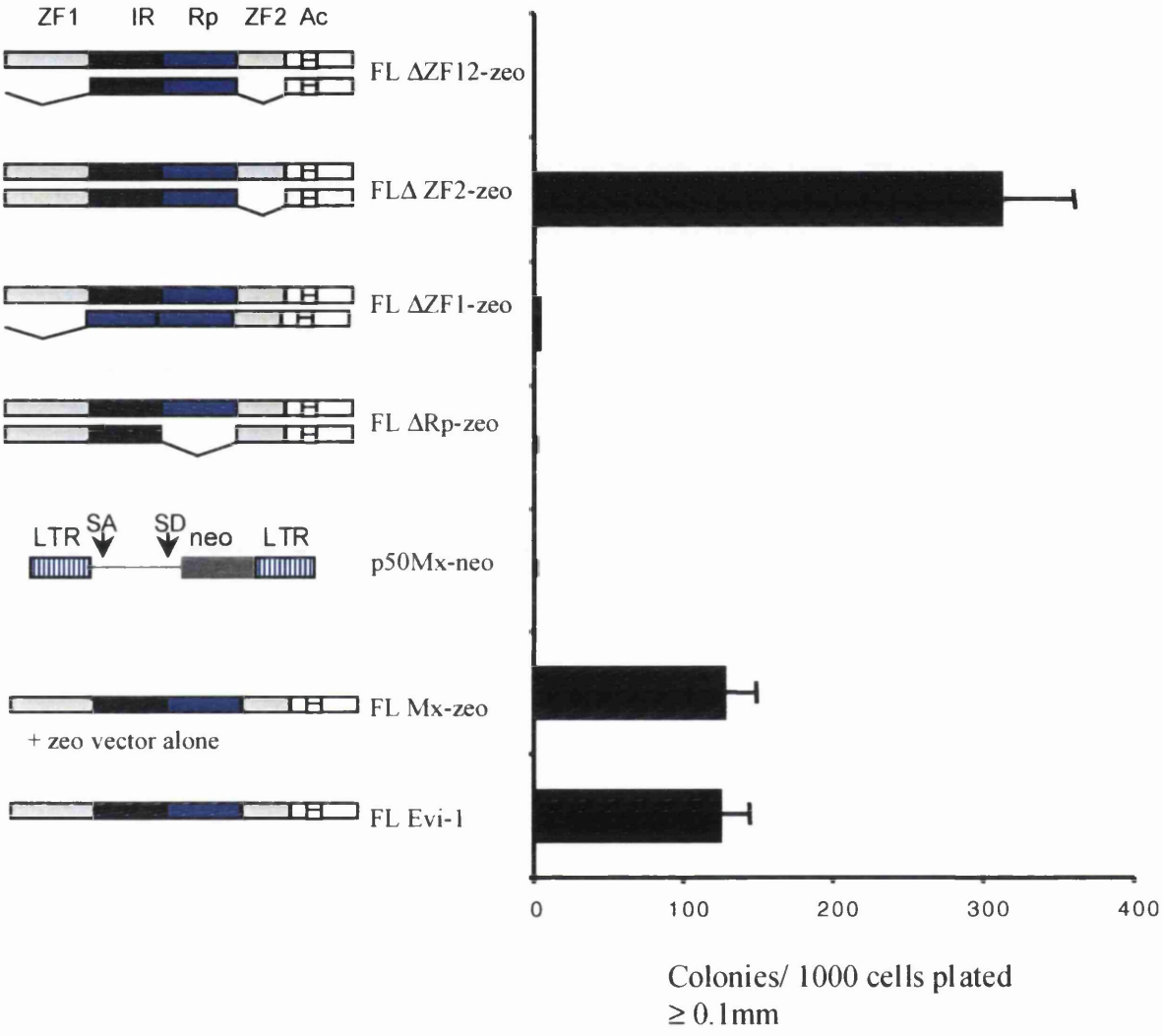


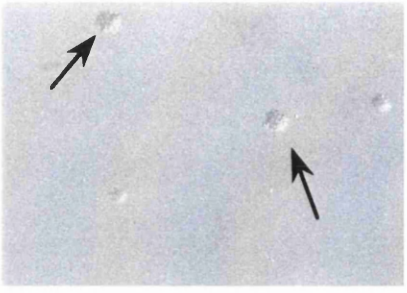
Figure 22: Morphological Analysis of Evi-1 Transformed Rat1 Fibroblasts Compared to Evi-1 Dominant Negative Revertants in Soft Agar Assays.

A) Typical control plates show transformed colonies formed from Rat1 cells expressing both p50FLEVI-1neo and p50MXzeo.

B) The dominant negative example shown here is transformed colonies emerging from Rat1 cells infected with p50MXFLevi-1neo and p50MΔRpzeo (the repressor mutant).

C) Aggressively transformed colonies formed from Rat1 cells expressing p50MFLevi-1neo and p50MΔZF2zeo (mutants lacking the second DNA binding domain). (N.B. The Rat1ΔZF2 mutant clones look no different to Rat1FL clones at confluence).

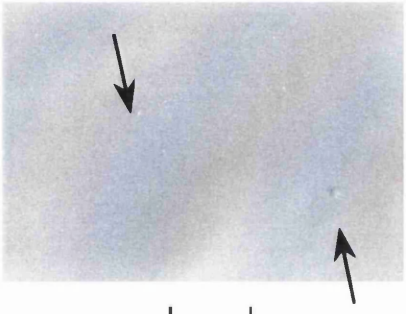
A)



1cm

TYPICAL CONTROL PLATE OF EVI-1 TRANSFORMED RAT1 FIBROBLASTS

B)



1cm

TYPICAL DOMINANT NEGATIVE PLATE

C)

1cm

CLONES FROM RAT1 CELLS CO-EXPRESSING FLEVI-1 AND ZF2 PROTEINS

Figure 23: Potential Models of Dominant Negative Action

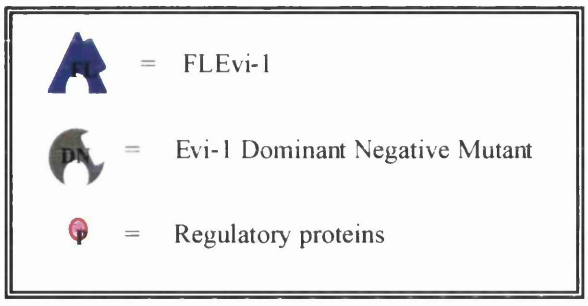
A) Normal evi-1 pathway involved in transformation utilises both additional accessory factors and DNA binding.

B) "Quenching" of evi-1 DNA binding Sites by deletion mutant proteins block the transformation pathway.

C) "Sequestration" of cofactors necessary for evi-1 regulation by evi-1 deletion mutants.

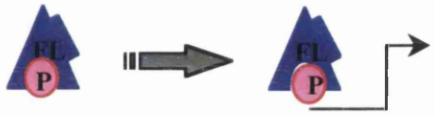
D) Heterodimerisation of evi-1 deletion mutants with FLevi-1 proteins altering the stiochiometry of the FL Protein and therefore inhibiting FLevi-1 regulation.

Where blue triangular shapes represent FLevi-1, grey circular shapes represent Dominant Negative Proteins and pink circles are accessory proteins/cofactors.



Transformation Pathway Functional

A) Normal Pathway

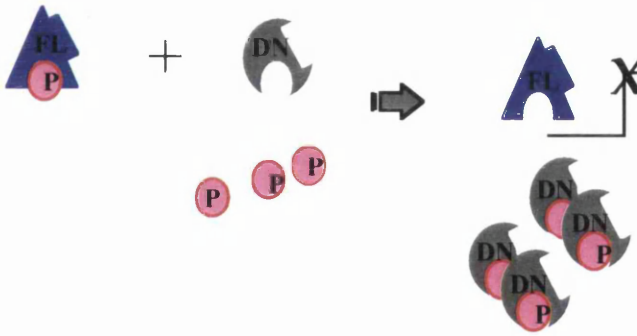


+++

B) "Quenching" of DNA Binding Activity

-

C) "Squelching" of Accessory Factors of Evi-1 Regulation



-

D) Heterodimerisation

-

Chapter 5: Protein-Protein Interactions Mediate Biological Activity of Evi-1

Recently, several sequence-specific repressor proteins have been found to reside in cells as components of large protein complexes, associating with co-repressors to regulate their biological activity. Furthermore, the pathogenesis of several haematopoietic disorders has been attributed to abnormal interactions between oncogenic proteins and nuclear co-repressors (Lin et. al., 1998, Grignani et. al., 1998 and Lutterbach et. al., 1998). Several co-repressors involved in this mode of so-called "transrepression" have been identified including Groucho (Fisher and Caudy , 1998), SMRT/NCoR (Chen et al., 1995 and Horlein et al., 1995) and CtBP (Turner and Crossley, 1998). Recent studies have demonstrated that Evi-1 associates with the short-range transcriptional co-repressor murine C-terminal Binding Protein 2 or mCtBP2 (Turner and Crossley 1998). Interestingly, both mammalian and *Drosophila* CtBP proteins are instrumental in modulating diverse biological processes. Human CtBP1 (hCtBP1) diminishes the tumourigenicity of adenovirus E1A (Schaeper et. al., 1998) and the *Drosophila* homologue, dCtBP1 has been identified as a key mediator of transcriptional repression by *Hairy*, *Snail*, *Knirps* and *Enhancer of split* during development (Nibu et. al., 1998). The recognition motif, **P-DLS-K** found in mammalian, viral and *Drosophila* proteins, mediates interactions with CtBP co-repressors (Turner and Crossley et. al., 1998). Evi-1 has two similar motifs, **PFDLTTK** and **PLDLSNG** located between amino acids 553-559 (CtBP1) and 584-590 (CtBP2) within the repressor domain (Rp).

Preliminary studies in our lab implicated a role for CtBP co-repressors in the biological activity of evi-1. First, mCtBP2 interactions with the Rp of evi-1 were confirmed using the yeast-2-hybrid assay (Dr. C. Bartholomew, unpublished data). Second, northern blot analysis revealed the expression of endogenous mCtBP2, in both kidney 293 cells and Rat1 fibroblasts (Dr. C. Bartholomew unpublished data - Figure 24), where evi-1 biological activities have been demonstrated. Third, evi-1 ΔCtBP mutants were created by PCR mediated site directed mutagenesis which substituted D, L amino acids for A, S within the P-DLS-K CtBP motifs of evi-1. These studies showed that evi-1 mutant proteins with ΔCtBP1, ΔCtBP2 or ΔCtBP1/2 mutations relieved the evi-1 Rp/mCtBP2 interaction in yeast-2-hybrid assays (Bartholomew, unpublished data - Figure 25).

The unexpected function of DNA-binding defective derivatives of evi-1 as dominant negative mutants of FLevi-1 transforming activity (described in Chapter 4 of this thesis) substantiated the proposal that these evi-1 mutants were able to recruit additional regulatory factors to mediate this inhibitory activity. Therefore from the cumulative evidence we hypothesised that the evi-1 oncogene could recruit CtBP2 co-repressors to mediate its repressor and oncogenic activities. It was hoped that these investigations would not only provide clues to evi-1 dominant negative function, but as a consequence would further disclose the intricacies of EVI-1 function in general.

5.1 Evi-1 mutants with a Partial Deletion of Rp, encompassing both CtBP sites, are Non-Transforming

Since two CtBP recognition motifs were recently discovered within in the repressor domain of evi-1 (Turner and Crossley et al., 1998), the transforming activity of evi-1 mutants with a 119 amino acid deletion encompassing both CtBP sites was examined. This mutant retrovirus, p50MΔRp514-633, was obtained from C. Bartholomew. Rat1 populations expressing p50MΔRp514-633 were isolated (See Western Blot analysis—Figure 26A) and introduced into the Rat1 transformation assay (for method overview see Figure 13). Deletion of this portion of evi-1 resulted in a dramatic reduction in macroscopic colony production in soft agar assays (Figure 26B).

5.2 Evi-1/CtBP Interactions Modulate Transcriptional Repression by Evi-1

Deletion mutagenesis studies have shown that the repressor domain of evi-1, localised between amino acids 514 to 724, could inhibit lexAVP16 mediated transcriptional activation of the adenovirus E1b minimal promoter in kidney 293 cells. In addition, optimal repressor activity required specific interactions of either the evi-1 ZF1 DNA binding domain or heterologous GAL4 DNA binding domains (GAL4DBD) (Bartholomew et. al., 1997). This repressor assay was also used to investigate the repressor activity of evi-1ΔCtBP deletion mutants. The pSG424 vector consists of the GAL4 (1-147) DNA binding domain (GAL4DBD) alone. The entire evi-1 Rp domain

was fused to pSG424 and this would provide our positive control. Likewise, pSG424 was also fused to the Rp domain with a mutated first CtBP site, the Rp domain with a mutated second CtBP site and Rp domain with both CtBP sites mutated. These vectors were designated pGWTRp, pGΔCtBP1, pGΔCtBP2 and pGΔCtBP1/2 respectively. The assays examined repression of the VP16 transactivator, which is fused to the DNA binding domain of lexA. The promoter activity was determined by relative CAT (Chloroamphenicol transferase) activity of the pL8G5CAT reporter (Hollenburg et. al., 1995) which has the E1b TATA box with eight copies of the lexA recognition sequence and five copies of the GAL4 UAS upstream of the CAT gene.

Figure 27 shows the results from these studies, where three separate transfections were carried out for each repressor construct. Transient transfections in 293 cells conferred low CAT activity, which is dramatically induced in the presence of lexAVP16. However, when the pGWTRp is also transfected, a marked 80% reduction in CAT activity is observed. Mutating the first CtBP site within the repressor domain had minor effects on repressor activity with pGΔCtBP1 mutants reducing CAT activity by 70%. Mutating the second site or both sites severely disrupts repressor activity with pGΔCtBP2 and pGΔCtBP1/2 mutants inhibiting CAT activity by only 30% and 35% respectively. This demonstrated that CtBP binding was necessary for transcriptional repression by evi-1—with the second CtBP site being more critical than the first for this activity.

5.2 Evi-1/CtBP Interactions Modulate Evi-1-mediated Transformation

The recombinant retroviral vectors used in these experiments were made by Dr. C. Bartholomew and included evi-1 mutants lacking the first CtBP binding site (p50MΔ CtBP1), evi-1 mutants lacking the second CtBP binding site (p50MΔCtBP2) and evi-1 mutants which have both CtBP sites removed (p50MΔCtBP1/2). The Rat1 transformation assay (described in Chapter 4) was used to determine the importance of evi-1/CtBP interactions for evi-1 oncogenic activity in Rat1 fibroblasts. Rat1 populations were established which contained p50MXneo, p50MFLevi-1neo, p50MΔCtBP1neo, p50MΔCtBP2neo or p50MΔCtBP1/2neo. Figure 28A shows Western blot analysis, with an anti-evi-1 specific antibody, of the respective wild type and mutant evi-1 proteins expressed in these cell populations. This analysis revealed the populations produced similar levels of the expected sized proteins. These cell lines were plated out into soft agar assays (in triplicate for each cell line) and their abilities to form macroscopic colonies were assessed after 3 weeks in culture. Figure 28B shows that evi-1 proteins with a mutated first CtBP site partially retain the ability to transform Rat1 fibroblasts, with ΔCtBP1 mutants producing 50% of the transforming activity of FLevi-1 controls. These studies also show that transformation is absolutely dependent on the second CtBP site of evi-1, with ΔCtBP2 mutants reducing colony production by 97% compared to FLevi-1 controls. Moreover, mutating both CtBP-binding sites (ΔCtBP1/2 mutants) of evi-1 also abolished the transforming activity of the FLevi-1 protein, with a 96%

reduction in transforming activity compared to FLevi-1 controls. As expected, Rat1 cell populations infected with the control virus p50Mxneo did not grow in soft agar. These results were reproduced in two separate experiments and both illustrated that the first CtBP site in Rp is partially required, and the second CtBP site in Rp is absolutely essential, for evi-1-mediated Rat1 transformation.

5.3 Discussion

5.3.1 The Integrity of CtBP sites is Required for Evi-1-Mediated Transcriptional Repression

The repressor domain of evi-1 encompasses two putative binding sites for the CtBP family of co-repressors. Therefore, it was hypothesised that evi-1 may directly interact with CtBP proteins to function as a transcriptional repressor. Mutating the first CtBP site within the Rp of evi-1 retained most of the evi-1 repressor activity (Figure 27). However, when the second CtBP site or both CtBP sites were altered, a significant fraction—although not all—of the repressor activity was lost (Figure 27). This shows that the second CtBP site was essential for recruiting CtBP co-repressor proteins, which enhance the repressor activity of evi-1. The retention of a small portion of this activity suggests that alternative mechanisms of repression might operate. Perhaps the mode of repression by evi-1 depends on the context of the targeted promoter.

5.3.2 Evi-1/CtBP interactions Modulate Evi-1-Mediated Cell Transformation

Partial deletion of the evi-1 Rp region required for CtBP binding (amino acids 514-633) has been found to significantly deplete the transforming activity of evi-1 in Rat1 fibroblasts (Figure 26). Subsequently, studies showed that the 514-633 region of evi-1 is required for binding the mCtBP2 co-repressor protein (Bartholomew, unpublished). Taken together, these observations suggest a model where co-repressor function mediates the transforming activity of evi-1. Mutating the first, second or both CtBP sites had profound effects on binding activity in yeast (Figure 25 - Bartholomew, unpublished). However, it was noted that mutating the second or both CtBP sites produced the greatest effects. In these studies, retroviral vectors expressing evi-1 CtBP binding-defective mutants were constitutively expressed in Rat1 cells and selected populations examined in soft agar assays. Evi-1ΔCtBP1 mutants produced only half of the macroscopic colony growth seen with FLevi-1 controls (Figure 28). Constitutively expressing evi-1ΔCtBP2 and evi-1 ΔCtBP1/2 mutants in Rat1 fibroblasts completely abolished transforming activity of evi-1 to levels comparable to vector only controls (Figure 28). Therefore, the magnitude of the effects seen for the specific CtBP mutants in Rat1 transformation correlated with that of DNA binding activity in yeast. Furthermore, these results suggest that the integrity of the second CtBP site in evi-1 was not only essential for repressor activity, but also crucial for evi-1-mediated transformation.

5.3.3 Possible Mechanisms of CtBP Action

The CtBP proteins are short-range co-repressors, which do not bind DNA but are likely to bridge the interaction of the evi-1 repressor with its ultimate target. CtBP may augment repression by 1) direct interactions with the basal machinery to assist in the recruitment of this machinery to the promoter, or 2) through interactions with the chromatin template, which may serve to modulate the accessibility of the template.

5.4 Summary

Interactions of evi-1 with the CtBP co-repressors facilitate evi-1 repressor function. However our results suggest that evi-1 can utilise other mechanisms for repressor activity. Furthermore mutating the second CtBP binding site or both CtBP sites, residing in the evi-1 Rp domain, can completely abolish evi-1 mediated transformation. Therefore, these data provide evidence for the molecular basis for both transcriptional repression and transformation by evi-1.

Chapter 5 Figures

Figure 24: Northern Blot Analysis of Rat1 and 293 cells with mCtBP2 (Dr. C. Bartholomew, unpublished)

To examine whether mCtBP2 proteins are expressed in 293 and Rat1 cell lines where evi-1 repressor and oncogenic activities have been demonstrated, Northern blot analysis of 20μg of total RNA from 293 (Lane 1) and Rat1 (Lane 2) cells was carried out with an mCtBP2 probe. A control GAPDH blot is also shown.

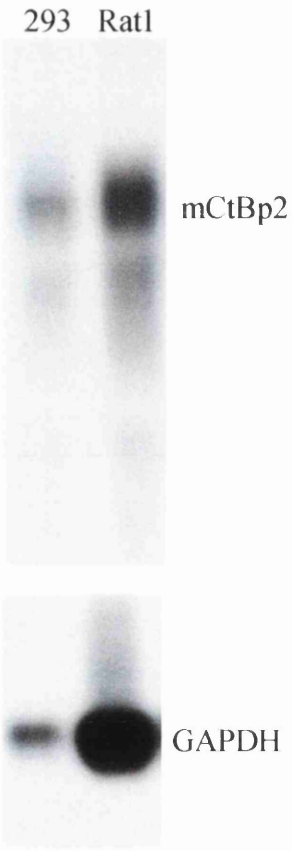

293
Rat1
mCtBp2
GAPDH

Figure 25: Interaction of mCtBP2 with WT and Mutant Evi-1 Repressor Domains (Dr. C. Bartholomew, unpublished)

Using the yeast-2-hybrid assay the importance of evi-1 CtBP binding sites in the evi-1/mCtBP2 interaction (in *Saccharomyces cerevisiae* SFY526) has been demonstrated. The graph opposite shows the interaction of the Rp domain of evi-1with mCtBP2. GAD (white box) and DBD (black box) refer to the activation and DNA binding domains of GAL4 respectively. The evi-1 wild type Rp domain (blue box), CtBP mutated regions (**X**) and mCtBP2 (grey box) are also shown.

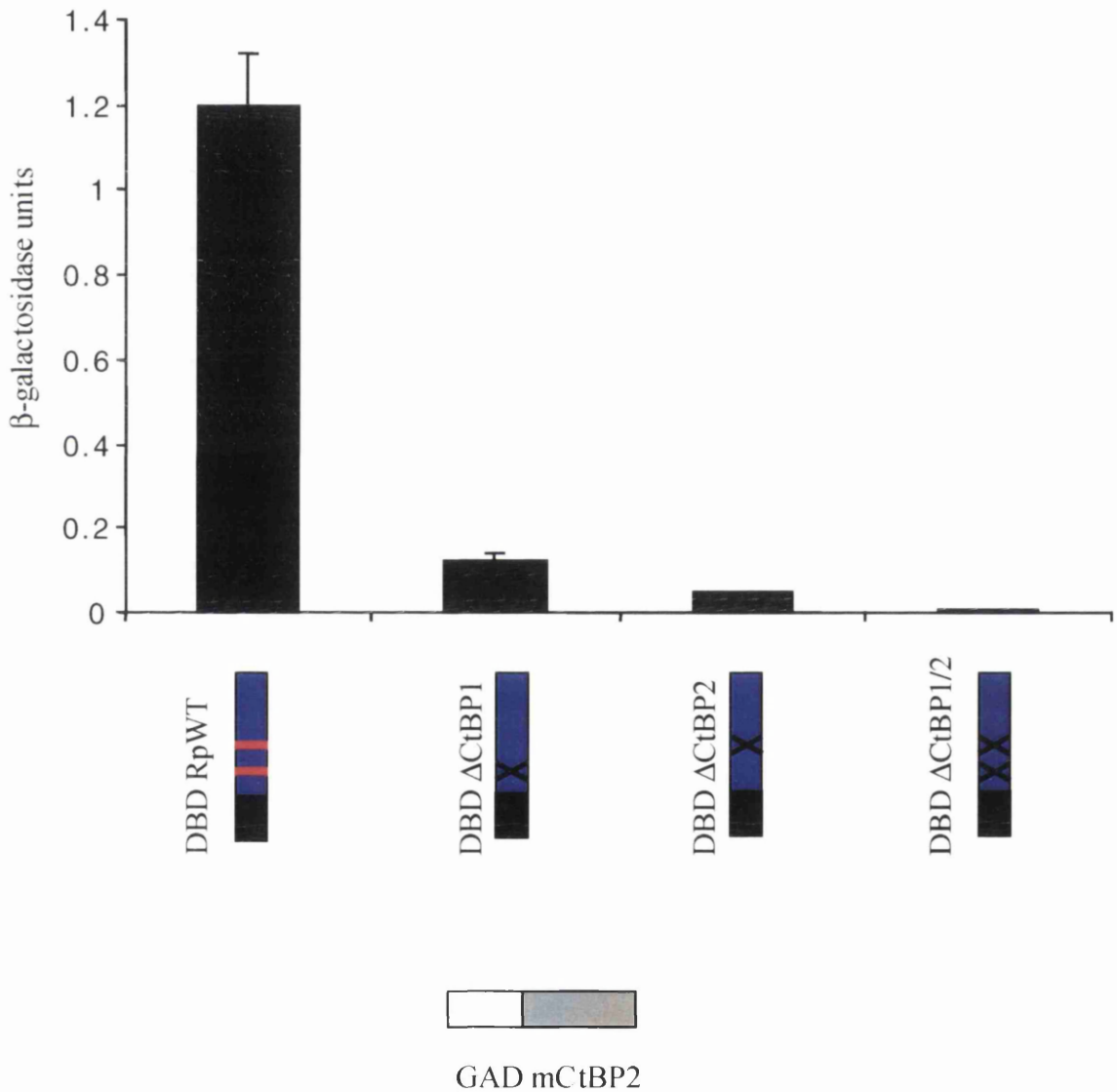
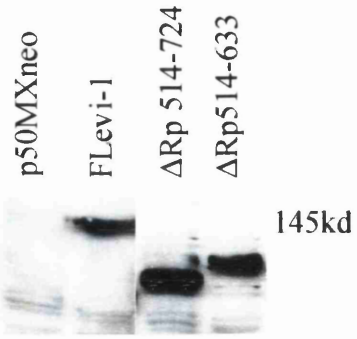

β-galactosidase units
1.4
1.2
1
0.8
0.6
0.4
0.2
0
DBD RpWT
DBD ΔCtBP1
DBD ΔCtBP2
DBD ΔCtBP1/2
GAD mCtBP2

Figure 26: Transformation of Rat1 Fibroblasts by Evi-1 Rp Domain Deletion Mutants

Previous deletion mutagenesis studies have shown that deletion of the region within Evi-1 containing putative CtBP sites reduces Evi-1s ability to transform Rat1 fibroblasts.
A) Western Blot analysis. Proteins were fractioned on a 7.5% SDS-polyacrylamide gel subjected to Western blot analysis using a specific anti-Evi-1 polyclonal antibody (1806). Lane 1 (-) is a Negative control of Rat1 cells transfected P50MXneo vector only. Lane 2 (+) is a Positive control of Rat1 cells transfected with p50FLEvi-1neo. Lane 3 is Rat1 populations constitutively expressing p50MΔRpWTneo deletion mutants lacking the entire repressor domain (ΔRp514-724). Lane 4 is Rat1 populations constitutively expressing p50M ΔRp514-633neo mutants lacking the section of repressor domain containing both putative CtBP binding sites (ΔRp514-633).

B) Macroscopic colony growth (>0.3mm) in soft agar assays by constructs expressing either P50MXneo vector alone, FLEvi-1, ΔRpWT mutants or ΔRp514-633 mutants are shown. Results are the mean ± standard deviation of triplicate plates examined for each population. Functional domains of the Evi-1 protein are shown: grey box, DNA binding domains; black box, intervening region; blue box, repressor domain; stripped box, acidic domain. The long terminal repeats (LTR-blue/white striped box) and neomycin resistance gene (dark grey boxes) of the retroviral vector are also indicated and potential CtBP binding sites are also shown (red oval shape) .

A)


p50MXneo
FLevi-1
ΔRp 514-724
ΔRp514-633
145kd

B)

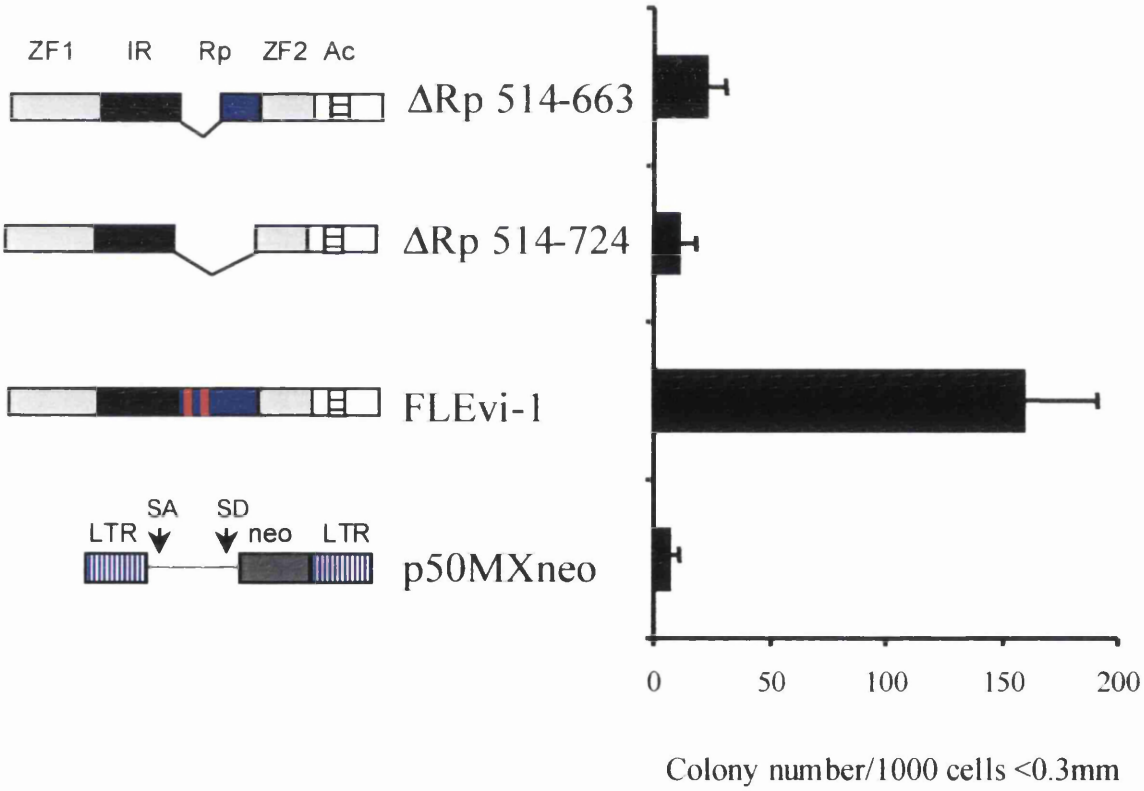
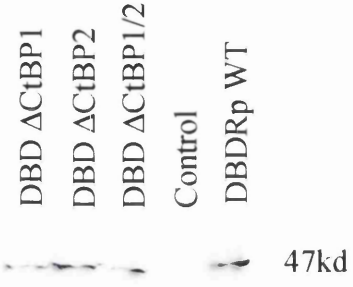

ZF1
IR
Rp
ZF2
Ac
ΔRp 514-663
ΔRp 514-724
FLEvi-1
SA
SD
LTR
neo
LTR
p50MXneo
0
50
100
150
200
Colony number/1000 cells <0.3mm

Figure 27: Repressor Activity of Evi-1 CtBP Binding Mutants

To determine the importance of Evi-1/CtBP interactions for Evi-1 repressor activity 2.5μg of either L8G5CAT and 1μg pHSVβgal were transiently transfected in the absence (-) or presence (+) of 25ng of LexAVP16 with 1μg of the wild type repressor, repressor CtBP mutants, or pSG424 alone as indicated. CAT activity is corrected for β-galactosidase activity in each sample. These experiments were performed on two occasions. Typical results are shown opposite: the results are the mean ± standard deviation of three independent transfections. For colour coding description refer to Figure 25.

A)


DBD ΔCtBP1
DBD ΔCtBP2
DBD ΔCtBP1/2
Control
DBDRp WT
47kd

B)

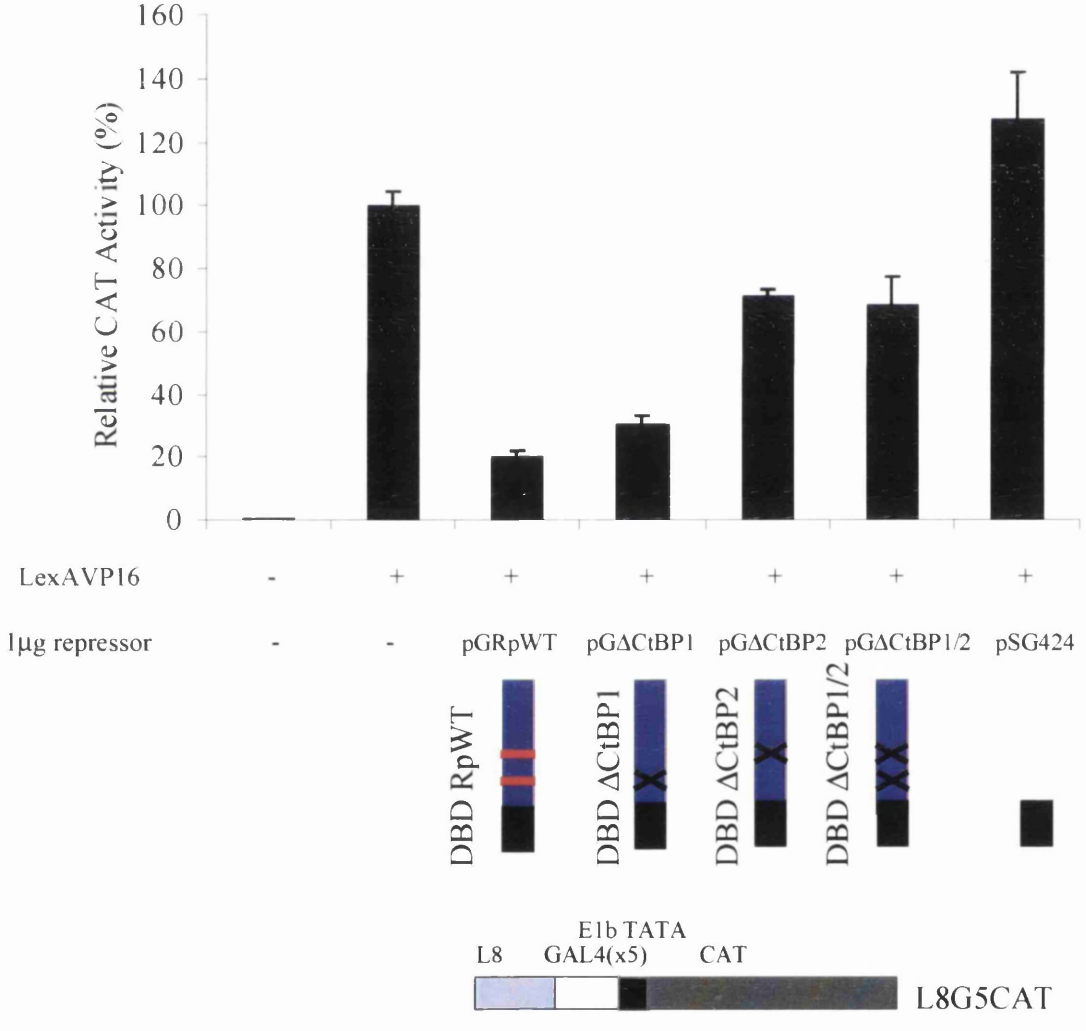

Relative CAT Activity (%)
160
140
120
100
80
60
40
20
0
LexAVP16 - + + + + + +
1μg repressor - - pGRpWT pGΔCtBP1 pGΔCtBP2 pGΔCtBP1/2 pSG424
DBD RpWT
DBD ΔCtBP1
DBD ΔCtBP2
DBD ΔCtBP1/2
E1b TATA
L8
GAL4(x5)
CAT
L8G5CAT

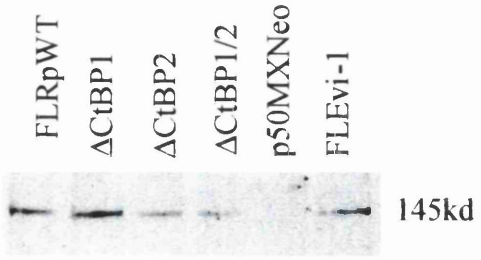
Figure 28: Rat1 Cell Transformation by Evi-1/CtBP Mutant Proteins

The transforming activity of Evi-1 ΔCtBP mutants is shown overleaf.

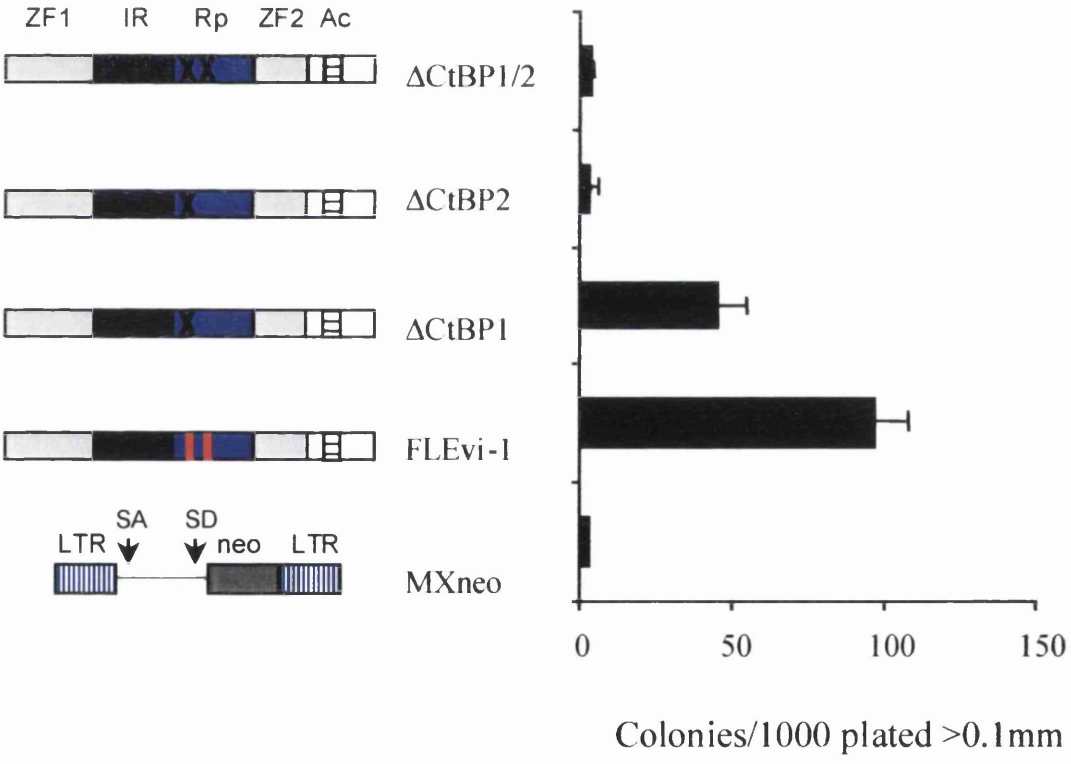
A) Western Blot analysis. Proteins were fractioned on a 7.5% SDS-polyacrylamide gel subjected to Western blot analysis using a specific anti-Evi-1 polyclonal antibody (1806). Lane 1 (-) is a Negative control of Rat1 cells transfected P50MXneo vector only. Lane 2 (+) is a Positive control of Rat1 cells transfected with p50FLEvi-1neo. Lane 3 is Rat1 populations constitutively expressing p50MΔCtBP1neo mutants which have an alteration in the first Evi-1 CtBP binding site. Lane 4 is Rat1 populations constitutively expressing p50M ΔCtBP2neo mutants which have an alteration in the second Evi-1 CtBP binding site. Lane 5 is Rat1 populations constitutively expressing p50MΔCtBP1/2neo mutants which have alterations in both Evi-1 CtBP binding sites.

B) These constructs were introduced into the Rat1 Transformation Assay to assess their transforming abilities in soft agar. The graph opposite illustrates the results from one representative population expressing each vector tested. Results shown are the mean ± standard deviation of the triplicate plates examined for each clone. Two independent Rat1 populations were examined for each vector and the same trends were observed. For colour coding description refer to Figure 26B.

A)


FLRpWT
ΔCtBP1
ΔCtBP2
ΔCtBP1/2
p50MXNeo
FLEvi-1
145kd

B)


ZF1
IR
Rp
ZF2
Ac
ΔCtBP1/2
ΔCtBP2
ΔCtBP1
FLEvi-1
SA
SD
LTR
neo
LTR
MXneo
0
50
100
150
Colonies/1000 plated >0.1mm

Chapter 6: Investigating the Biological Activity of Evi-1 in Haematopoietic Cells

Aberrant overexpression of EVI-1 is frequently observed in patients with acute myeloid leukaemia, chronic myeloid leukaemia in blastic crisis and myelodysplastic syndromes. The transformed phenotype of acute myeloid leukaemia is characterised by a loss of normal controlled proliferation and a differentiation block leading to the production of immature blasts cells instead of the development of fully mature haematopoietic progenitors. It is postulated that ectopic EVI-1 expression disrupts the normally "highly orchestrated" process of haematopoiesis and through unknown mechanisms helps to maintain the transformed leukaemic state. Consistent with this, constitutive evi-1 expression in primary erythroid progenitor cells (Kreider et al., 1993) and 32Dcl3 cells (Morishita et al., 1992) blocks erythropoietin (EPO) and granulocyte colony stimulating factor (G-CSF) responsiveness respectively, resulting in the cessation of the proliferation, differentiation or survival of these haematopoietic progenitors.

Others have shown that full length evi-1 (FLevi-1) can transform Rat1 fibroblasts by permitting growth of macroscopic colonies in soft agar (Kurokawa et al., 1995). Deletion mutagenesis studies show that the second DNA binding domain (ZF2) (Kurokawa et al., 1995), the repressor domain (Rp) (Bartholomew et al., 1997) and the first DNA binding domain (ZF1) (Kilbey and Bartholomew, 1998) are all required for optimal Rat1 transformation. These findings are reproduced in Chapter 4 of this thesis.

Until now no studies have addressed which of these regions, critical for optimal Rat1 transformation, are essential to the oncogenic function of EVI-1 in myeloid cell transformation. As a means to help dissect the complex pathway of transformation by evi-1 in haematopoietic cells, an experimental system was established whereby retrovirally-transduced haematopoietic progenitors could be examined in methyl cellulose colony assays.

6.1 Development of a Cell Culture System for the Clonal Differentiation of Retrovirally-Transduced Murine Primary Bone Marrow Cells

To study the role of evi-1-mediated transformation in the pathogenesis of myeloid leukaemias an assay was established where retrovirally-transduced haematopoietic progenitors of several lineages could be examined in colony assays (see Methods section 2.2.1). This provided a useful system to study the biological activity of both normal and mutant evi-1 proteins in haematopoietic cells. Figure 29 shows a schematic diagram of the assay. In brief, the kidney epithelial packaging cell line, Bosc23 are transfected with the retroviral vector of interest. The following day these cells are irradiated to halt their growth. Immediately after irradiation, each flask of virus-producing cells is co-cultivated with bone marrow cells flushed from one CBA/Ca mouse femur. These cells are incubated together for 48 hours, for gene transfer to take place, then plated out in methyl

cellulose. These colony assays provided evidence for the presence of progenitor cells that are able to produce single and multi-lineage colonies of differentiated cells. In these studies, colonies are defined as greater than 50 cells and are morphologically distinct. The three colony types scored in these assays were colony forming unit-granulocyte macrophage (CFU-GM), burst forming unit-erythroid (BFU-E) and colony forming unit-macrophage (CFU-M). Examples of these colonies are seen in Figure 30. The CFU-GMs (Figure 30A) are characterised by their typically dense core of cells surrounded by a peppering of individual cells around the periphery. A BFU-E colony can be seen in Figure 30B. These colonies appear as erythroid clusters or "bursts" ranging from transparent to brick red, depending on the haemoglobinisation status. Figure 30C shows a typical macrophage colony which arise as a loose scattering of individual bone marrow cells.

Giemsa-stained cytospin preparations of these colonies verified colony assignment. Photographs of cytospins are shown in Figure 31A, 31B and 31C. Figure 31A shows erythroblastic islands of maturing erythroid cells. Cytospin 31AI reveals an abundance of mature normoblast cells. The arrow in Figure 31AII shows fully mature red blood cells where nuclei are being expelled and haemoglobin synthesis is complete. Figure 31B shows typical CFU-M colonies. The cytoplasm of these large cells is often highly vacuolated and they can contain degenerated cell debris. Figure 31C shows CFU-GM cytospins, which reveal a wide range of maturing granulocyte and macrophage cells: the black arrows indicate mature "banded" or "segmented" neutrophils.

6.1.1 Retroviruses Used in Bone Marrow Assays

All of the retroviruses to be utilised in these studies are illustrated in Figure 32. In addition to FLevi-1 (p50MFLevi-1neo) and Δ324 (p50MΔ324neo) natural evi-1 isoforms, deletion mutants lacking the Rp domain (p50MΔRpneo), the ZF1 domain (p50MΔ ZF1neo), theZF2 domain (p50MΔZF2zeo) and both ZF1 and ZF2 domains of evi-1 (p50MΔZF12zeo) were examined. Retroviral titres (also seen in Figure 32) were determined by infecting semi-confluent NIH-3T3 cells with serial dilutions of viral supernatant followed by a selection in G418 or zeocin® depending on the vector.

*6.2 Production of Haematopoietic Colonies in Primary Bone Marrow Cells Infected with FL*evi-1

Initially, the effect of enforced FLevi-1 expression on haematopoietic colony formation was investigated. Bone marrow harvested from 4-week-old CBA/Ca mice was cocultivated with Bosc23 cells producing either p50MXneo (negative control) or p50MFLevi-1neo viruses. Bone marrow cells cultured without virus were also used as an additional control. Following the two-day co-cultivation infected cells were assayed in the colony forming assays. Haematopoietic cells derived from 3 femurs were infected with each virus. Cells infected with p50FLevi-1neo vector showed an 80% reduction in CFU-GM production and a 90% reduction in BFU-E production, compared with the p50MXneo controls (Figure 33). CFU-M colonies were unaffected in both FL-infected

cells. These experiments were repeated in more than six separate occasions and the same trend was observed each time.

6.3 Production of Hameatopoietic Colonies in Primary Bone Marrow Cells Infected with Evi-1 *Deletion Mutants*

Over-expressing FLevi-1 results in a block in CFU-GM and BFU-E colony production in murine bone marrow cells (section 6.2). To elucidate whether certain functional domains of evi-1 were required for this biological activity, various evi-1 deletion mutants were introduced into the experimental system described in section 6.1. The effects of constitutively expressing deletion mutant constructs lacking the Rp domain, the ZF1 domain, theZF2 domain and both ZF1 and ZF2 domains of evi-1 were examined. The alternative splice form of evi-1, Δ324 was also tested. As before, bone marrow was harvested from 4-week-old CBA mice. Bosc23 cells producing either FLevi-1 (positive control), p50MXneo (negative control), p50MXzeo (negative control) or the aforementioned evi-1 deletion mutants were cocultivated with bone marrow cells flushed from independent murine femurs. Bone marrow cells cultured without virus were also used as an additional control. After a 48-hour co-cultivation, infected cells were then assayed in the colony formation assays. Each retrovirus was infected into 3 femurs taken from different mice. The haematopoietic cells infected with FLevi-1 vector showed a dramatic 80% reduction in CFU-GM and 90% reduction in BFU-E compared to vector controls. Cells infected with Δ324, ΔRp, ΔIR or ΔZF1 mutants disrupted CFU-GM and

BFU-E colony production by similar quantities to the FL protein (Figure 34). Expressing evi-1 mutants lacking the ZF2 domain also results in a drop in CFU-GM and BFU-E colony production but the effects were not as remarkable. CFU-GM colonies are reduced by 60% and BFU-E colony production by 50% in this case. These effects were also abated in assays with mutants lacking both DNA binding domains (ΔZF12 mutants). The results indicated that constitutive expression of ΔZF12 mutants reduced CFU-GM colony formation by 40% and the appearance of BFU-E colonies was only reduced by 25%. These experiments were repeated in three or more separate experiments and the same trends were observed each time. The transforming activities of all evi-1 retroviruses examined in Rat1 cells are compared to the relative reductions in BFU-E and CFU-GM colony production in Table 2.

6.4 Evaluation of Gene Transfer Efficiency of Neo^r Retroviral Vectors

Examination of proviral integration into bone marrow colonies by PCR analysis allowed: (1) verification that all retroviruses used were inserted into the genome of each progenitor cell type examined and (2) estimation of viral infection efficiency. A PCR-based approach was utilised for this purpose and DNA extracts from colonies were prepared from a *Stratech* bone marrow-specific DNA extraction kit. Two rounds of PCR were performed on DNA extracts prepared from pooled (5 colonies) and individual bone marrow colonies. The first round of PCR used external primers which amplified a 1250bp region, which included most of the neo gene and a section of a polylinker site

within the p50MXneo vector (See Figure 35). A second round of PCR with nested primers was used to increase the sensitivity of this procedure. It was reasoned that the major analysis of proviral integration should be on CFU-M colonies since CFU-M colony production appeared unperturbed upon expression of all retroviruses. Infection efficiencies of the zeocin resistant vectors were not examined here.

Figure 36 shows the agarose gel electrophoresis photographs of the second round PCR products. The varying intensities of PCR amplified DNA products may reflect differences in colony size, and therefore infected cell number, originally plucked from methyl cellulose assays. These results are also tabulated in Table 3. Analysis of fifty individual p50MXneo-transduced CFU-M colonies revealed 50% of colonies were positive for p50MXneo. Five individual CFU-M colonies infected with either FLevi-1 or evi-1 mutant viruses ranged from 20-80% positive for p50MXneo, respectively. Concerns raised over the PCR-sensitivity, with respect to the detection of retrovirus from such small cell numbers, resulted in a portion of the colonies being pooled. Indeed, pooling five colonies/sample generally resulted in higher values. Moreover, PCR analysis of p50MXneo-infected cells revealed that this vector inserts into progenitors of all cell types examined. No uninfected (control) colonies were positive for the p50MXneo virus. The Low sample numbers, analysed for colonies transduced with FL and mutant vectors compared to p50MXneo, may explain why in some cases the percentage of unpooled colonies exceeds that of pooled colonies for a particular vector type.

6.5 Discussion

6.5.1 Enforced Expression of Evi-1 Blocks the Production of Erythroid and Granulocyte-Macrophage Colonies in Primary Bone Marrow Cells

These studies have shown that constitutively active FLevi-1 provokes a block in both erythroid and granulocyte-macrophage production. A compensatory increase of other lineages was not observed here, suggesting lineage switching was not involved. FLevi-1 expression reduced both CFU-GM and BFU-E production by approximately 80%. However, macrophage colony formation was not affected. Interestingly, the haematopoietic cell line MID+ , which expresses the evi-1 gene at high levels, maintains the capacity to differentiate to macrophages in the presence of IL-6 (Morishita and Ihle, unpublished data cited in Kreider et. al., 1993). This is consistent with our finding that evi-1 expression has no effect on macrophage production.

Results from these studies are consistent with previous findings that inappropriate evi-1 expression in primary erythroid progenitor cells (Kreider et al., 1993) and the myeloid leukaemia cell line, 32Dcl3 (Morishita et al., 1992) disrupts the responsiveness of the cells to erythropoietin resulting in a cessation of erythroid development. However, the studies described here are the first to demonstrate a block in granulocyte-macrophage colony production in primary bone marrow cells by constitutive evi-1 expression, although it has been previously reported that enforced expression can block Granulocyte

Colony Stimulating Factor (G-CSF) induced survival of 32Dcl3 cells (Khanna-Gupta et. al., 1996).

The observations, reported in this chapter, do suggest that one role for EVI-1 in a leukaemic programme is to induce a block in erythroid and myeloid development or to impede the survival of these cells at a stage prior to BFU-E and granulocyte-macrophage stages, respectively. Two potential models for leukaemia progression involving constitutive EVI-1 activation are (1) EVI-1 overexpression is one of the first mutations within these neoplasms mediating a differentiation block. However, additional genetic alterations are required to contribute to the proliferation and/or survival of haematopoietic cells in leukaemias. (2) The block of colony production observed here may reflect EVI-1 expressing cells' need for the support of the haematopoietic microenvironment to survive.

6.5.2 Enforced Expression of Evi-1 Deletion Mutants Impairs Haematopoietic Progenitor Production

Surprisingly, retroviral vectors lacking the Rat1-transforming ΔIR mutants and the Rat1-nontransforming Δ324, ΔRp and ΔZF1 mutants all seemed to act in an identical fashion to FL evi-1 proteins (Figure 34/Table 2): over-expression in primary bone marrow also resulted in reduction in CFU-GM and BFU-E colony formation. Similar to FLevi-1 infected cells, CFU-GM and BFU-E reductions ranged from 80-90% compared to vector only controls. It is intriguing that domains, proven to be essential for transformation of

Rat1 fibroblasts, appeared to have no influence on the biological function of evi-1 in bone marrow cells.

Retroviral vectors expressing mutants lacking the ZF2 domain were not as effective at blocking CFU-GM and BFU-E production (Figure 34). CFU-GM reductions were near 60% and BFU-E reductions of 50% were seen, which suggested that the second DNA binding domain could be important for this biological activity. Indeed, mutants lacking both ZF1 and ZF2 domains also block CFU-GM and BFU-E growth with reduced efficiencies (Figure 34). However, viral titres of the p50MΔZF2zeo and p50MΔZF12zeo constructs were notably lower than those evaluated for the other viruses, which may explain the somewhat mediocre abilities of these mutants in blocking colony production. However, the ΔZF2 and ΔZF12 vectors encode zeocin® resistance and all other vectors encode G418 resistance. Therefore, it is impossible to compare the zeo vector and neo vector titres from NIH3T3 focus formation assays since the zeocin selection is a much harder selection than G418.

Introducing a collection of evi-1 deletion mutants into haematopoietic cells results in an identical phenotype to FLevi-1 proteins. The results suggest that optimal activity requires the ZF2 domain as ΔZF2 and ΔZF12 mutants but not ΔZF1 mutants have a reduced effect on colony formation.

6.5.3 Testing the Experimental System

6.5.3.1 Viral Titre Analysis

Evaluation of viral titres (Figure 32) by focus formation of virus-infected NIH3T3 cells showed p50MXneo titres generally exceeded p50MFLevi-1neo titres 10 fold and mutant titres 20 fold.

6.5.3.2 PCR Analysis

From the data it was observed that the production of erythroid and granulocyte-macrophage colonies was not completely abolished by FLevi-1, suggesting that the remaining colonies arose from cells not infected with retrovirus. Therefore, the bulk of the PCR analysis was carried out on CFU-M, CFU-GM and BFU-E bone marrow colonies transduced with p50MXneo vector alone. We were able to conclude that all three colony types can be infected with the p50MXneo retrovirus (Figure 36). Proviral integration studies on the FLevi-1 and mutant retroviruses were limited by their small sample size and the inability to analyse CFU-GM and BFU-E colonies (owing to the effect these vectors have on production of these colonies). However, the analysis did prove that these retroviral vectors could also infect macrophage progenitors.

Indeed, from the viral titre data, overall infection efficiencies were much higher than expected. However, these data cannot be compared because they involve completely different methods: focus formation from supernatant infection and infection of bone marrow cells in a co-cultivation with virus producer cells, respectively. Finally, preparing

DNA extracts from (and therefore PCR) minute numbers of cells may have limited detection of retrovirus by this PCR technique.

To obtain a clearer view of retroviral expression in haematopoietic cells of all constructs future studies must examine RNA expression levels of all of the vectors by RT-PCR analysis.

6.5.4 Possible Mechanisms of Evi-1 Action in Haematopoietic Cells

6.5.4.1 Distinct Domains are Required for the Biological Activity of Evi-1 in Rat1 Cells and Haematopoietic Cells

These studies have demonstrated that the biological activity of FLevi-1 in haematopoietic cells is not altered by the removal of Rp and ZF1 domains, which are both essential for evi-1s oncogenic activity in Rat1 fibroblasts. Mutants lacking both DNA binding domains ZF1 and ZF2 or the ZF2 domain only, suggest that evi-1 may be partially dependent on the ZF2 domain. These mutants have reduced biological activities compared to FLevi-1, but do not restore erythroid and myeloid differentiation levels to control levels. Therefore, there may be an unexamined region residing in the remainder of the protein (the C-terminus) that is absolutely essential for the disruptive effects of evi-1 overexpression in haematopoietic cellular development.

*6.5.4.2 High Level Expression of FL*evi-1 *and Evi-1 Deletion Mutants Could Induce Cell Death in CFU-GM and BFU-E Progenitors*

The possibility that introducing these proteins into bone marrow cells simply kills these cells should not be ruled out. It is conceivable that each of these constructs could overstimulate an evi-1 regulatory pathway leading to cell cycle arrest, senescence or necrosis of the cells. Studies showing that Rat1 fibroblasts modified to constitutively express evi-1 induce cell cycle progression and confer a growth advantage on these cells (Kilbey et al., 1999) would tend to discount this theory. However, there are several examples where the level of a particular oncogene appears to regulate the fate of cells. For example, low levels of Raf activity will actuate the progression of the cell cycle, whereas stronger activation results in cell cycle arrest (Woods et al., 1997). Similarly c-Myc induces proliferation or apoptosis, depending on the concentration of serum (Evan et. al., 1992).

*6.5.4.3 FL*evi-1 *and Evi-1 Deletion Mutants Dominantly Disrupt the Regulation of Factors Essential for Normal Haematopoiesis*

Constitutive expression of FLevi-1 and evi-1 deletion mutants could block differentiation pathways by physically interacting with key haematopoietic cell regulators of the erythroid and myeloid lineages. There is now a widely accepted view of the combinatorial action of transcription factors. This notion has grown to accommodate the idea that factors themselves form large multiprotein complexes or possibly even "lineage-specific machines" within the scheme of haematopoiesis (Cross and Enver, 1997). For example, studies have shown that LMO2 may form a transactivating complex in erythroid

cells that includes SCL, GATA-1, E2A and LDB1/NL1 (Visvader et. al., 1997). Constitutively expressed EVI-1 is likely to corrupt key regulatory factors required for the proper development of the erythroid and myeloid lineages.

It appears that GATA-1 facilitates erythropoiesis by recruiting factors into multiprotein complexes and therefore GATA-1 is a potential target for EVI-1 protein-protein interactions. Furthermore, EVI-1 has a GATA consensus sequence in the ZF1 domain and reporter assays have shown that (1) EVI-1 can bind to a GATA-like binding sequence through ZF1 and (2) EVI-1 blocks GATA-1-mediated trans-activation (Kreider et. al., 1993 and Kreider et. al., 1994 respectively). That fact that no known physiological GATA-1 sites can be bound by EVI-1 has questioned the physiological importance of these findings. Furthermore, we have shown here that a block in erythroid differentiation does not require the ZF1 domain.

An additional possibility is that EVI-1 can homodimerise. Studies examining the possible self-association of EVI-1 are of great importance because such interactions could facilitate the regulation of EVI-1 function. Acidic extensions are typical dimerisation domains of proteins (Chen and Bieker, 1996 and Geiman et al., 2000) and the acidic domain of evi-1 is still intact in all the constructs used in these studies. Therefore FLevi-1 and evi-1 deletion mutants infected into these cells could dimerise with endogenous evi-1 proteins. Consequently, this may interfere with myeloid and erythroid differentiation pathways that may be regulated by endogenous FLevi-1 proteins.

6.6 Summary

In closing, the experimental system described provides an in vitro approach using primary bone marrow cells to assay the biological function of the *evi-1* oncogene. Inappropriate expression of FLevi-1 and the evi-1 the deletion mutants examined either blocked the production of erythroid and myeloid lineages or somehow impede the survival of these cells at a stage prior to BFU-E and CFU-GM stages, respectively. An intermediate phenotype was achieved when overexpressing ΔZF2 and ΔZF12 mutants but not ΔZF1 mutants of evi-1 in this biological system. This suggests that the ZF2 domain may have been partially required for this biological activity in haematopoietic cells.

The exact mechanism of evi-1 biological action in bone marrow cells was unclear from these studies. This issue is likely to be unravelled when the normal role for EVI-1 in the commitment and lineage determination of haematopoietic cells is fully investiagted. It would be useful to investigate the role of evi-1 in haematopoiesis within the context of an intact animal. Since evi-1 knockouts have proved lethal to mice (Hoyt et al., 1997), mouse chimaeras could be created by generating evi-1 $^{+/-}$ and evi-1 $^{-/-}$ embryonic stem cell clones. Single cell suspensions could be prepared from haematopoietic organs at different stages, which could be examined in methyl cellulose colony assays to determine the morphology and frequency of erythroid and myeloid progenitors in the organs of mutant foetuses compared to phenotypically normal littermates. Therefore, evi-1 mutant mouse chimaeras could provide us with a clearer picture of EVI-1 function in haematopoiesis, which would undoubtedly have implications for the consequences of

constitutive *EVI-1* activation observed in myeloid leukaemias.

Chapter 6 Figures

Figure 29: Methodology of Bone Marrow Colony Assays

See text for details.

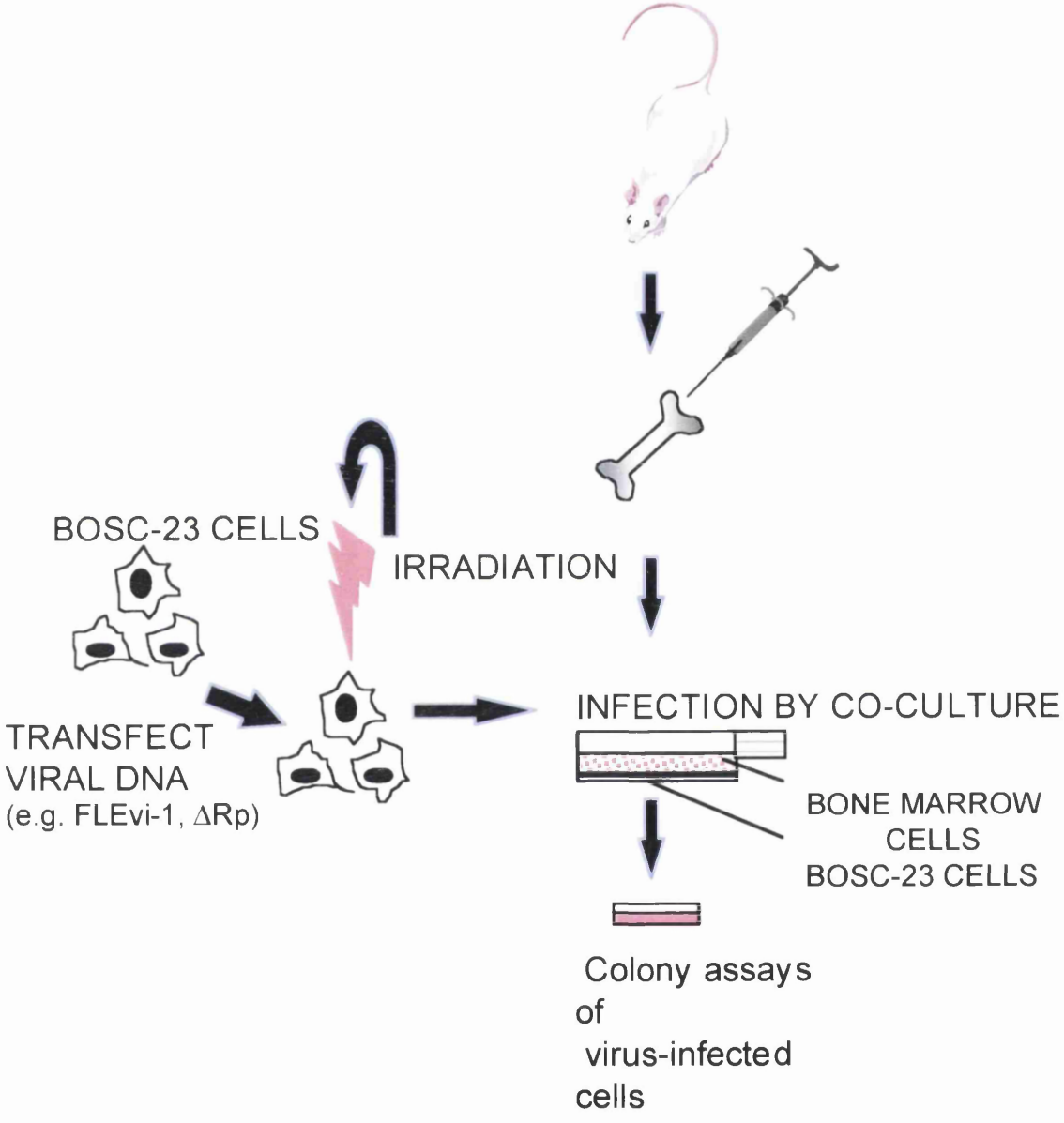
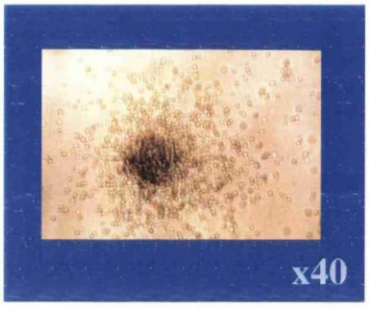

BOSC-23 CELLS
IRRADIATION
TRANSFECT
VIRAL DNA
(e.g. FLEvi-1, ΔRp)
INFECTION BY CO-CULTURE
BONE MARROW
CELLS
BOSC-23 CELLS
Colony assays
of
virus-infected
cells

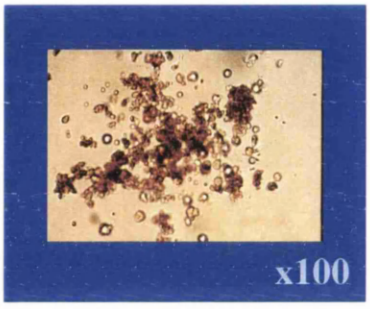
Figure 30: Morphological Analysis of Haematopoietic Colonies

Examples of haematopoietic colonies scored in these assays are shown. Colony forming Unit -Granulocyte Macrophages (or CFU-GMs) are shown in A (40x Magnification). A typical Burst forming unit-Erythroid colony (or BFU-E) can be seen in B (100x Magnification). C shows a typical macrophage colony (40x Magnification).

A) CFU-GM



B) BFU-E



C) CFU-M

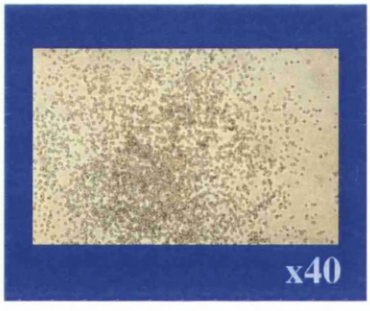
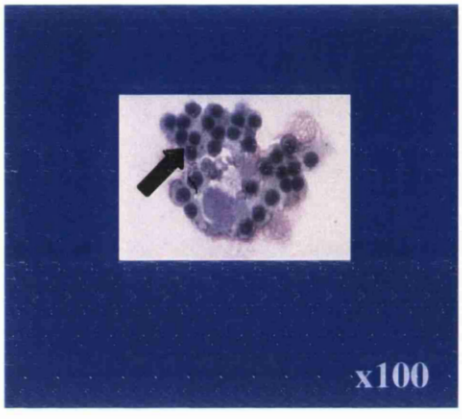


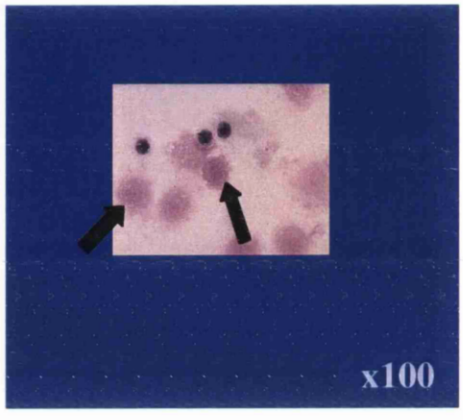
Figure 31: Morphological Verification of Colony Assignment by Examination of Cytospin Preparations

A) Cytospin preparations of Burst Forming Unit-Erythroid colonies. See text for details.

I
Control BFU-E

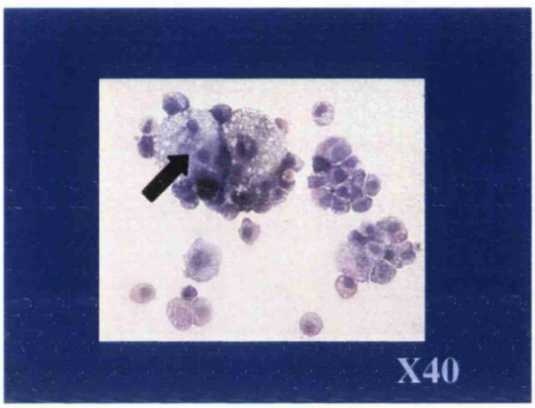


II
ΔZF12 Mutant BFU-E

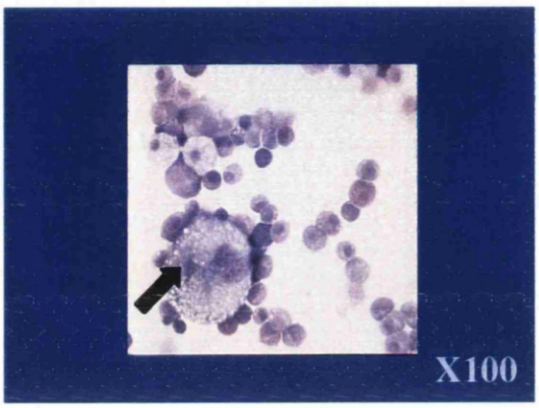


B) Cytospin preparations of Colony Forming Unit-Macrophage colonies. See text for details.

I
Control MAC



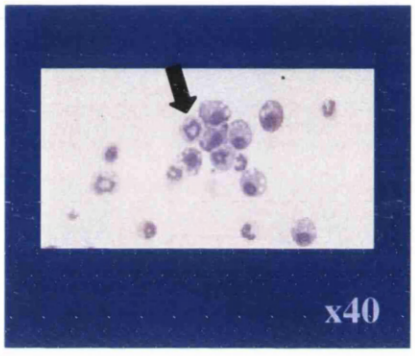
II
Repressor Mutant MAC



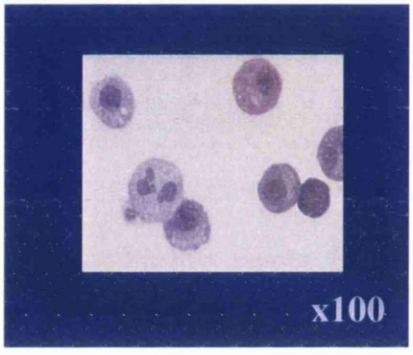
C) Cytospin preparations of Colony Forming Unit-Granulocyte Macrophage colonies.

See text for details.

I
Control GM



II
Full Length GM



III
ΔZF12 Mutant GM

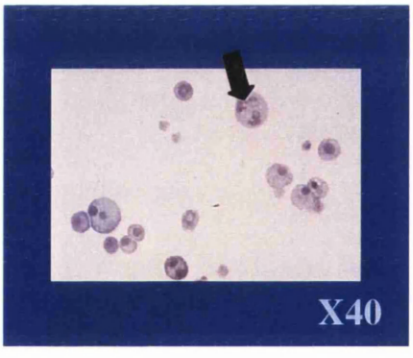


Figure 32: Schematic Representation of Retroviral Vectors used in Primary Bone Marrow Studies

Evi-1 functional domains are shown with the DNA binding domains (light grey boxes), the intervening region (black boxes), the repressor domain (dark blue boxes) and the acidic domain (black/white striped boxes). The p50MXneo vector is also shown indicating the long terminal repeat (LTR) (blue/white striped box) and the neomycin/zeocin resistance gene (dark grey boxes).

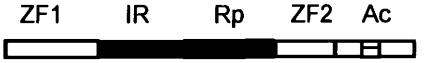
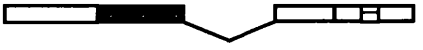
Viral Titres	Vector	
$4.6x10^5$	p50MXneo	
		
$1.9x10^4$	FLneo	
$4.9x10^3$	Δ324neo	
$6.3x10^3$	ΔRpneo	
$3.9x10^3$	ΔIRneo	
$4.7x10^3$	ΔZF1neo	
$1x10^2$	ΔZF2zeo	
$1x10^2$	ΔZF12zeo	

Figure 33: Production of Erythroid, Granulocyte-Macrophage and Macrophage Colonies in Primary Bone Marrow Cells Infected with FLevi-1

The graph shows haematopoietic colony formation in methyl cellulose of virus-infected primary murine bone marrow cells. Formation of BFU-E, CFU-GM and CFU-M are shown for mock-infected cells and cells infected with retrovirus p50MXneo (control) or p50MFLevi-1neo. Three individual femurs taken from individual mice were infected for each vector used. Infected cells from each femur were plated out in triplicate into methyl cellulose cultures. These experiments were repeated more than 4 times. The histogram opposite illustrates results from a typical experiment: the mean ± standard error of the mean is shown in each case. For colour coding key see Figure 32 legend.

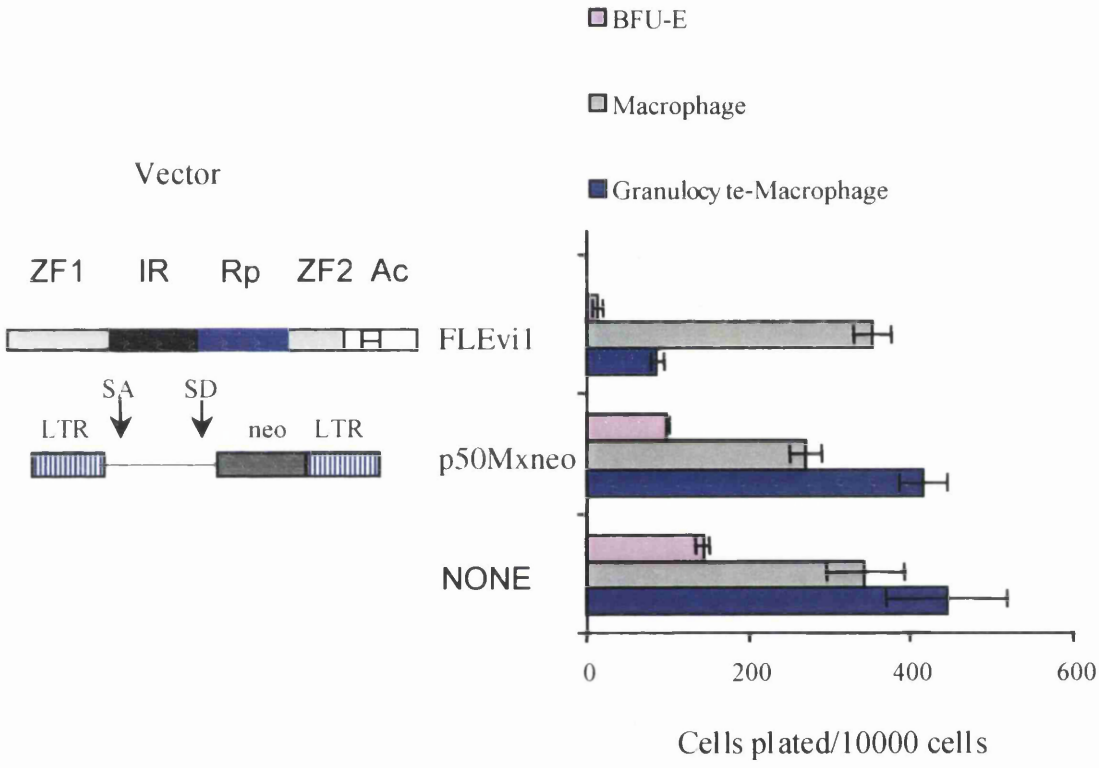

BFU-E
Macrophage
Granulocy te-Macrophage
Vector
ZF1
IR
Rp
ZF2
Ac
FLEvi1
SA
SD
LTR
neo
LTR
p50Mxneo
NONE
0
200
400
600
Cells plated/10000 cells

Figure 34: Colony Assays of Murine Bone Marrow Cells Infected with FLevi-1 and Evi-1 Mutant Retroviruses

The histogram shows haematopoietic colony formation in methyl cellulose after gene transfer by co-cultivation of marrow with packaging cell lines independently producing one of the following retroviruses: p50MXneo (neo vector controls), p50MXzeo(zeo vector controls), p50MFLevi-1neo, p50MΔ324neo, p50MΔRpneo, p50MΔIRneo, p50MΔZF1neo, p50MΔZF2zeo or p50MΔZF12zeo. Bone marrow cells cultured without virus were used as an additional control. Three femurs taken from individual mice were infected for each vector used. Again, infected cells from each femur were plated out in triplicate into methyl cellulose cultures. These experiments were repeated more than 4 times. The histogram opposite illustrates results from a typical experiment: the mean ± standard error of the mean is shown in each case. On the right of the graph the relative transforming activities of these constructs in Rat1 cells are denoted by + and -. For colour coding key see Figure 32 legend.

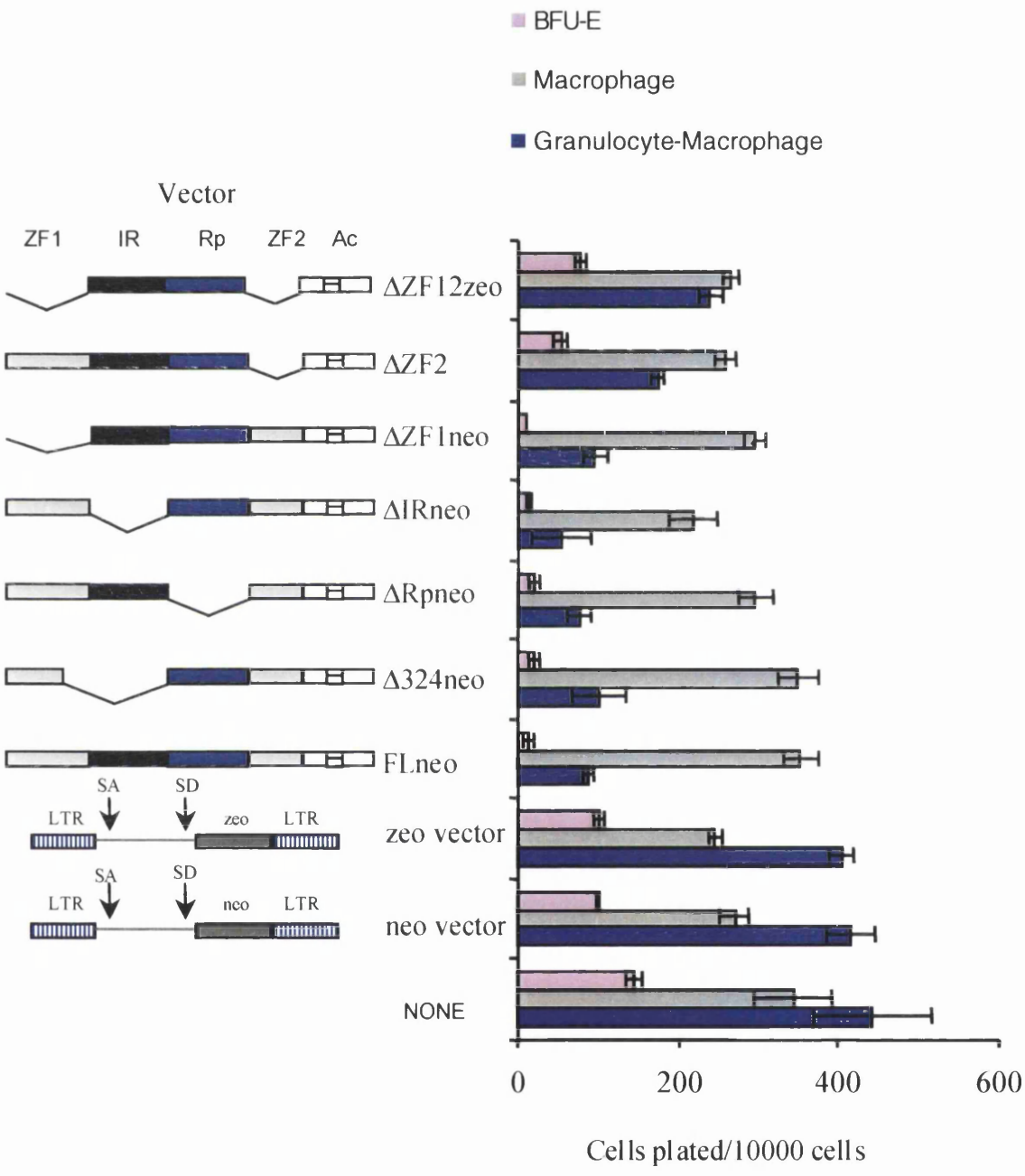

BFU-E
Macrophage
Granulocyte-Macrophage
Vector
ZF1
IR
Rp
ZF2
Ac
ΔZF12zeo
ΔZF2
ΔZF1neo
ΔIRneo
ΔRpneo
Δ324neo
FLneo
SA
SD
LTR
zeo
LTR
zeo vector
SA
SD
LTR
neo
LTR
neo vector
NONE
0
200
400
600
Cells plated/10000 cells

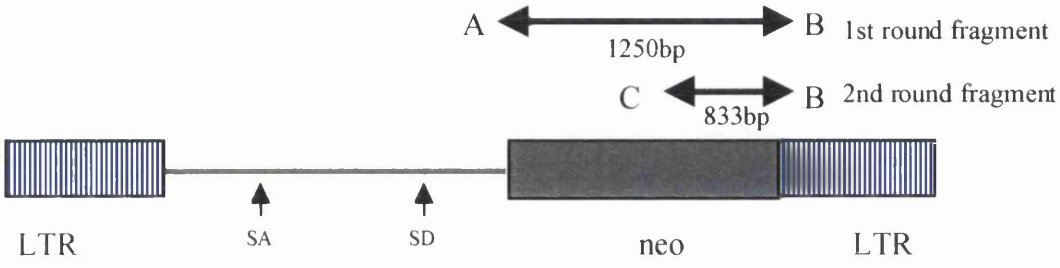
Table 2: Table of percentage reduction in erythroid and granulocyte-macrophage colony growth by FLevi-1 and Evi-1 deletion mutants

VECTOR	Percentage CFU-GM Reduction	Percentage BFU-E Reduction	Transformation In Rat1 Cells
p50MFLneo	79%	86%	+++
p50MΔ324neo	76%	80%	-
p50MΔRpneo	82%	80%	-
p50MΔIRneo	87%	87%	+++
p50MΔZF1neo	77%	90%	-
p50MΔZF2zeo	58%	48%	-
p50MΔZF12zeo	41%	25%	-
p50MXneo	0%	0%	-
p50MXzeo	0%	0%	-

Figure 35: Oligonucleotides used for Analysis of Proviral Integration into Bone Marrow Cells

Neo^r vectors were assessed for proviral integration of individual bone marrow colonies. Both the p50MXneo vector alone and the same vector expressing either FLEVI-1 or EVI-1 deletion mutant proteins were amplified using two rounds of PCR (See Methods Section 2.2.1). The double-headed arrows indicate approximate sites of primers used and the resultant product sizes for both rounds of PCR amplification. The 5'→3' primers were Neo-specific and the 3'→5' primer was specific to a region within a polylinker site of the p50MXneo vector. For colour coding key see Figure 32 legend.

p50MXneo



A = External I Neo	5' caa gat gga ttg cac gca gg 3'
B = External II Polsite	5' ctc gag gtc gac tct ag 3'
C = Internal neo A	5' aaa gta tcc atc atg gct gat 3'

Figure 36: PCR amplification of genomic DNA isolated from bone marrow colonies infected with p50MXneo and Evi-1 derivative vectors

PCR products visualised on a 0.8% agarose gel after the second round of PCR. Pink plus signs indicate samples that were positive in first round reactions. The ethidium bromide-stained product of an 833bp DNA fragment on each gel indicates specific amplification by p50MXneo-specific nested primers (for sequences see Figure 35 Oligos B and C). In the first half of each sample range colonies were pooled with 5colonies/pool. The remaining samples were individual bone marrow colonies.

SAMPLE RANGE	VECTOR	COLONY TYPE
1-100	p50MXneo	MAC (macrophages)
101-110	p50MXneo	BFU-E(erythroid cells)
111-120	p50MXneo	GRAN-MAC (granulocyte-macrophage)
121-130	p50MFLevi-1neo	MAC
131-140	p50MΔIRneo	MAC
141-150	p50MΔ324neo	MAC
151-160	P50MΔRPneo	MAC
161-170	NO VIRUS	4 MAC, 4 GRAN-MAC 3 BFU-E

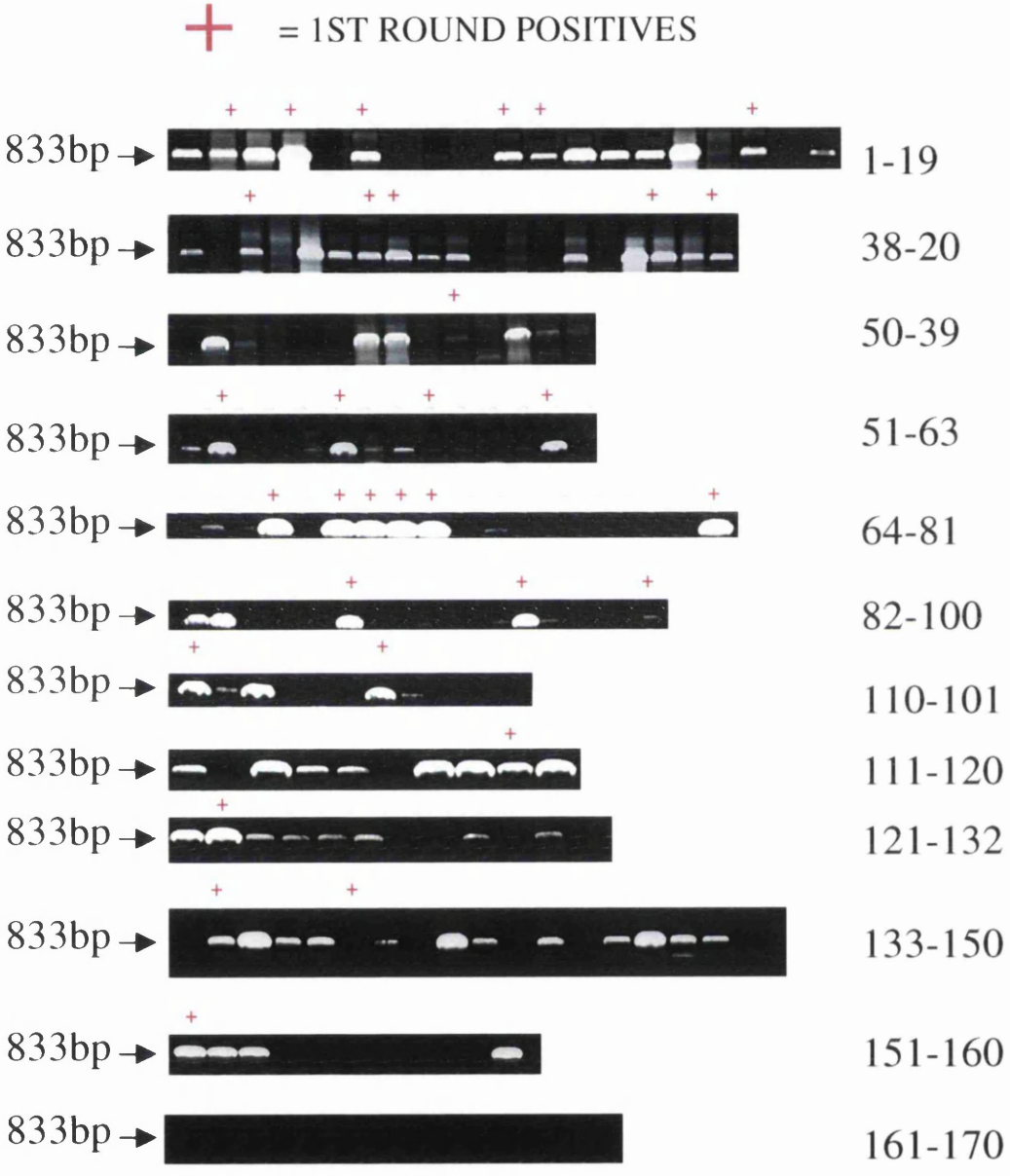

+ = 1ST ROUND POSITIVES
833bp
1-19
833bp
38-20
833bp
50-39
833bp
51-63
833bp
64-81
833bp
82-100
833bp
110-101
833bp
111-120
833bp
121-132
833bp
133-150
833bp
151-160
833bp
161-170

Table 3: Table of Transduction Efficiencies

The table shown opposite indicates the percentage transduction efficiencies for all the retroviruses examined in each colony type.

Vector	UNPOOLED COLONIES			POOLED COLONIES		
	CFU-M	CFU-GM	Bfu-E	CFU-M	CFU-GM	Bfu-E
p50MXneo	52%	80%	60%	74%	80%	40%
p50MFLneo	20%	_	_	100%	_	_
p50MΔ324neo	80%	_	_	60%	_	_
P50MΔRpneo	20%	_	_	60%	_	_
p50MΔIRneo	60%	_	_	60%	_	_
No Vector Controls	0%	_	_	0%	_	_

Chapter 7: Summary Discussion

Evi-1 deletion mutants have been created (Chapter 3), which act in a dominant negative fashion over full length evi-1 (FLevi-1) proteins in Rat1 fibroblasts constitutively expressing full length evi-1 (Rat1FLevi-1 cells) (Chapter 4). From the evidence presented it is suggested that these mutants utilise distinct mechanisms to mediate this inhibitory activity. That is, ΔZF1 and ΔRp mutants with partially and fully intact DNA binding domains respectively could inhibit transformation through a "blocking" mechanism where mutants compete with FLevi-1 for evi-1 DNA binding sites. On the other hand, ΔZF12 mutants may directly interact with and sequester proteins necessary for FLevi-1 regulation and thus inhibit the biological activity of FLevi-1. Since the initial studies described in Chapter 4 confirmed that the repressor domain is essential for the transformation of Rat1 fibroblasts, it was postulated that ΔZF12 mutants could "squelch" essential evi-1-regulatory proteins that directly interact with evi-1 through this region. Interestingly, the studies described in Chapter 5 demonstrated that two conserved motifs —to which the murine C-terminal Binding Protein 2 (mCtBP2) co-repressor protein binds —are crucial for evi-1-mediated repression and transformation in kidney 293 epithelial cells and Rat1 fibroblasts, respectively. Taken together, the results from Chapters 4 and 5 suggest that high levels of the ΔZF12 mutants could sequester endogenous mCtBP2 and in doing so, dominantly inhibit FLevi-1transforming activity in Rat1FLevi-1 cells. However, the possibility that other factors are involved cannot be discounted. An additional CtBP family member, mCtBP1 has similar binding specificities to mCtBP2

(Furusawa et al., 1999) and is therefore likely to interact with EVI-1 and mediate EVI-1 biological activity. However, potential evi-1/mCtBP1 interactions have not been examined here.

The precise mechanism by which CtBP might effect the repression of transcription is not clear. However, some studies have shown an interaction of CtBP1 with histone deacetylase (HDAC) (Sundqvist et al., 1998). Indeed, there is a common theme emerging whereby the recruitment of HDACs by oncogenic transcriptional repressors is essential to the progression of haematopoietic disorders. The biological role for co-repressor complexes in acute promyelocytic leukaemia is demonstrated in studies of the retinoic acid receptor alpha (RARα) fusion proteins. Recruitment of HDACs is indispensable for the capacity of acute promyelocytic leukaemia (APL) fusion proteins (PML-RARα and PLZF-RARα) to block myeloid differentiation and is responsible for the retinoic acid-resistant phenotype of PLZF-RARα APLs (Lin et al., 1998, Grignani et al., 1998). Similarly, the transcriptional repressor AML1-ETO fusion protein of AML recruits a HDAC complex through the ETO portion, which correlates with AML1-ETO inhibition of haematopoietic precursor differentiation (Lutterbach et al., 1998). The repressor function of these fusion proteins, like evi-1 appears to be central to their transforming potential. Therefore, it will be extremely interesting to determine if histone deacetylation also plays a role in evi-1 regulation perhaps via the CtBP interaction. An alternative mechanism of CtBP-mediated repression stems from recent observations that CtBP2 interacts with the human polycomb proteins (Sewalt et al.,1999). Polycomb proteins are important in repression of certain homeotic genes in *Drosophila* (reviewed in Jones et al.,

2000). Although the mechanisms of this repression are unclear, current models speculate that the (*Polycomb*-Group) *Pc*G proteins can package regions of DNA into heterochromatin-like complexes. This suggests that CtBP2 co-repressor can act as a bridging protein between sequence-specific DNA binding proteins and the *Pc*G complex.

The biological consequences of enforced evi-1 expression on haematopoietic cell production and the functional significance of evi-1 functional domains for evi-1 activity in primary bone marrow cells were also investigated (Chapter 6). It was shown that FLevi-1 and a selection of evi-1 deletion mutants all abrogated the production of erythroid and myeloid progenitors in methyl cellulose colony assays. Most intriguing was the observation that ΔZF12 mutants—defective in DNA binding—could also impede eyrthroid and myeloid production (although an intermediate phenotype was observed compared to FLevi-1 controls).

Potential mechanisms for this biological activity of evi-1 and evi-1 deletion mutants in haematopoietic cells are discussed in Chapter 6 section 6.5.4. However, there is also some evidence to suggest that EVI-1 has a normal role to play in haematopoiesis. Therefore, in addition the proposals in section 6.5.4, it is also possible that introducing FLevi-1 or evi-1 deletion mutants into haematopoietic cells deregulates endogenous FLevi-1 activity within these cells. Indeed, developmental studies have reported a hypocellularity phenotype of evi-1 knockout mice, which is consistent with a role for EVI-1 in general proliferation (Hoyt et. al., 1997). In more recent studies an embryonic stem cell model for haematopoietic differentiation also suggested a normal role for EVI-1

in differentiating haematopoietic cells (Wimmer et. al., 1998). The myelodysplastic syndrome 1 gene (MDS-1) has also been implicated in the progression of myeloid leukaemias. Interestingly, both EVI-1 and an *MDS1/EVI-1* isoform have been detected in both normal bone marrow and leukaemic cell lines (Sigurdsson et. al., 1997) and *MDS1/EVI-1* is also expressed in all tissues in which EVI-1 is expressed (Wimmer et. al., 1998). *MDS1/EVI-1* mRNA gives rise to a protein that contains an additional 188 amino acids at its N-terminus. This converts EVI-1 from a repressor of transcription into a transactivator (Solderholm et. al., 1997). Furthermore, EVI-1 can repress *MDS1/EVI-1* transactivation. EVI-1 and *MDS1/EVI-1* are expressed at low levels in normal bone marrow and it is postulated that any disturbance in a finely regulated equilibrium of these potentially antagonistic isoforms of EVI-1 may contribute to neoplastic growth. Therefore in the studies described in Chapter 6, overexpressing FLevi-1 in primary bone marrow cells could cause an imbalance in this equilibrium, resulting in mds1/evi-1 repression and consequently an impairment of erythroid and myeloid lineages. Indeed, studies have shown that the relative levels of *EVI-1* and *MDS1/EVI-1* favour *EVI-1* in the bone marrow of leukaemia patients with 3q26 rearrangements (Solderholm et al., 1997). Alternatively, evi-1 deletion mutants expressed at high levels could act as dominant negatives over low level endogenous EVI-1 or MDS1/EVI-1 proteins and produce the same disruptive effects. Moreover, having uncovered the critical nature of CtBP binding for the transformation of Rat1 cells by evi-1 it is possible that this dominant negative action is elicited through evi-1 deletion mutants "mopping up" endogenous CtBP proteins (or other factors) necessary for the endogenous functions of evi-1 in the bone marrow.

7.1 Future Applications

As discussed at the end of Chapter 6, future investigations examining a broad role for evi-1 in adult haematopoiesis are of paramount importance. However, the data from this thesis has collectively introduced some intriguing new twists concerning the specific mechanisms of EVI-1 regulation.

The discovery of the critical nature of evi-1/CtBP interactions for the biological activity of evi-1 (Chapter 5) may also provide mechanisms for artificially regulating gene expression. To examine the importance of the evi-1/CtBP interaction in haematopoiesis, chimaeric mice generated from FLevi-1 null mutant (-/-) ES cells and evi-1ΔCtBP null mutant (-/-) ES cells could be generated and the phenotypes of haematopoietic tissues compared at different stages of development. Also, since EVI-1 and the CtBP co-factor interact through a short peptide motif, it is tempting to speculate that small molecules could be developed which interfere with this interaction leading to the reactivation of certain EVI-1 target genes. Moreover, it would also be of interest to determine whether these molecules could revert the transformed phenotype of myeloid leukemia cell lines expressing EVI-1.

The evi-1 dominant negative mutants created (Chapter 3/Chapter4) could be conditionally expressed in myeloid leukemia cell lines constitutively expressing evi-1 to assess whether these mutants can revert the transformed phenotype of these cells. For example, a

tetracycline-regulated gene expression system in the 32Dcl3 evi-1-expressing myeloid leukaemia cell line could be created whereby the expression of a dominant negative mutant of evi-1 is under tetracycline-mediated regulation. The effects of continuous expression of this mutant on growth and spontaneous or growth-factor mediated differentiation could be examined. In 32Dcl3 cells differentiation induction is achieved by adding G-CSF growth factor. To assess the differentiation, proliferation and survival status of these repressor-mutant expressing cells genetic markers (lysozyme), cell cycle analysis (flow cytometry) and morphological markers (cytospins) could be examined.

Evi-1 expression was also found to block the production of erythroid and myeloid progenitors in primary bone marrow colony assays (Chapter 6). Moreover, deletion mutants, which were non-transforming in Rat1 colony assays produced indistinguishable effects from FLevi-1 in primary bone marrow assays. Whether proliferation, differentiation or apoptosis is blocked by these retroviruses could not be determined in these studies. If evi-1 was conditionally expressed in multipotential cell line such as FDCP-Mix, which can be induced to differentiation into various haematopoietic lineages, evi-1 could be switched on and the proliferation, differentiation and apoptotic status of the cells could be assessed using survival assays, differentiation markers and flow cytometry.

Normal haematopoietic regulation consist of many molecular protein links whose specific patterns of combinatorial action act to guide gene transcription and regulate commitment and lineage determination of haematopoietic cells. Furthermore, it is becoming clear that another level of regulation is superimposed on this pattern of combinatorial activation, which involves the repression of genes specific for one lineage by transcription factors promoting other lineages. Indeed, it is thought that this antagonism between transcription factors could provide the developmental switch in the choice between two lineages, thereby rendering lineage commitment irreversible and preventing ectopic expression of lineage-specific genes. Moreover, it is postulated that such mechanisms may be important in understanding the differentiation blocks that can contribute to leukemogenesis. Therefore, EVI-1 overexpression could block erythropoiesis or myleopoiesis by interactions with erythroid-specific transcription factors, such as GATA-1, SCL or LMO2 or myeloid-specific factors such as PU.1 or c-myb—all key regulators in the development of these lineages (See Chapter 1 section 2.2.1). EVI-1 would sequester these factors and consequently deregulate haematopoietic development. Co-immunoprecipiation experiments could examine these interactions *in vivo*. The future identification of such EVI-1 protein-protein interactions and indeed the findings demonstrated here that CtBP binding is crucial for evi-1-mediated transformation may have profound implications for the treatment of human neoplasias where EVI-1 is overexpressed. Indeed, the more such protein-protein interactions are revealed and their biological roles characterised, the greater the likelihood of interceding and altering the expression of genes—such as *EVI-1*—involved in the pathogenesis of human disease.

Chapter 8: References

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