IN VIVO ANALYSIS OF THE *ETV6-CBFA2* FUSION GENE

A thesis submitted for the degree of Doctor of Philosophy by

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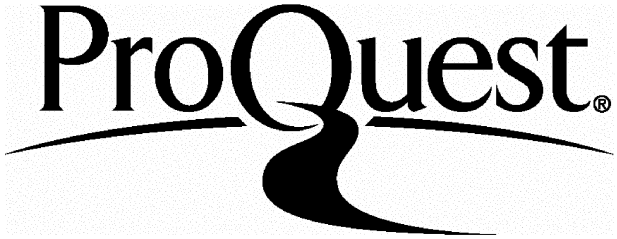

ABSTRACT

The *ETV6-CBFA2* (*TEL-AML1*) fusion gene arises from the chromosomal translocation, t(12;21)(p13;q22) which occurs in approximately 30% of childhood B-cell acute lymphoblastic leukaemia. The role of the fusion gene in leukaemogenesis was investigated by using a DNA construct containing the murine *Etv6* exon 5 fused in frame to human *CBFA2* (exon 2-8) to mimic the *ETV6-CBFA2* fusion identified in human acute lymphoblastic leukaemia. This DNA-targeting construct was introduced by homologous recombination into mouse embryonic stem cells and the fusion gene shown to be transcribed under the control of endogenous *Etv6* elements. High level chimeras were generated by blastocyst injection of targeted embryonic stem cells. The chimeras were subsequently crossed to generate offspring heterozygous for the *Etv6-CBFA2* mutation. To date, neither the chimeric animals (at 14.5 months) nor the heterozygous offspring (at 10 months) have demonstrated any overt phenotypic evidence of leukaemia. However, mice homozygous for the *Etv6-CBFA2* mutation died between dE10.5-11.5. The phenotype of these embryos was similar to that described in the *Etv6* knock-out model. Our study demonstrates that the *Etv6-CBFA2* fusion gene resulting from the t(12;21) translocation is not, by itself, oncogenic and we postulate that further genetic events are required for the development of leukaemia. Furthermore, the lethal phenotype observed in the mice carrying the homozygous disruption of the *Etv6* gene is likely to be due to a lack of the *Etv6* DNA binding domain.

TABLE OF CONTENTS

ABSTRACT **2**

TABLE OF CONTENTS **3**

TABLE OF FIGURES **7**

TABLE OF TABLES **9**

ACKNOWLEDGEMENTS **10**

ABBREVIATIONS **11**

INTRODUCTION **14**

1.1. Haematological Malignancy 14

1.1.1. Leukaemia 14

1.1.2. Malignant Lymphoma 15

1.2. Chromosomal Changes and Malignancy 15

1.2.1. Chromosomal Translocations 15

1.2.1.1. Gene Enhancement Via Antigen Receptor Gene Activation: The Burkitt's Lymphoma Model 27

1.2.1.2. Gene Fusion 27

1.2.1.2.1. Philadelphia Positive Leukaemia 28

1.2.1.2.2. The t(12;21) Translocation in Paediatric ALL 28

1.3. The Core Binding Factor Complex. 29

1.3.1. CBFA2 31

1.3.2. CBFA1 36

1.3.3. CBFA3 36

1.3.4. CBFβ 37

1.4. Chromosomal Translocations Involving the Core Binding Factor Complex 38

1.4.1. t(8;21)(q22;q22) 38

1.4.2. t(3;21)(q26;q22) 43

1.4.3. Inv(16)(p13;q22) 45

1.5. ETV6 48

1.6. Chromosomal Translocations Involving ETV6 50

1.6.1. t(12;21)(p13;q22) 50
1.6.2. t(5;12)(q33;p13) 52
1.6.3. t(9;12)(q34;p13) 54
1.6.4. t(9;12)(p24;p13) 54
1.6.5. t(1;12)(q25;p13) 55
1.6.6. t(12;15)(p13:q25) 55
1.6.7. t(12;22)(p13;q11) 55
1.6.8. t(3;12)(q26;p13) 56
1.6.9. Other Translocations Involving ETV6 56
1.7. Haematopoietic Development. 57
1.8. Experimental Studies to Investigate the Role Fusion Proteins Play in Tumourogenesis 59
1.8.1. Transgenesis 59
1.8.2. Generating a Null ("Knock-Out") Phenotype by Homologous Recombination 60
1.8.3. Introducing New Genetic Material ("Knock-In") by Homologous Recombination 60
1.9. Aim of Project 61
MATERIALS AND METHODS 63
2.1. Materials (see Appendix I) 63
2.2. Methods 63
2.2.1. DNA Preparation 63
2.2.1.1. Plasmid DNA (Minipreps) 63
2.2.1.2. Large Scale Plasmid Preparation (Maxi Prep) 64
2.2.1.3. Preparation of Phage Vector High Titre Stock (HTS). 65
2.2.1.4. Large Scale λ Bacteriophage Preparation 65
2.2.1.5. DNA Extraction from Tissue Samples 66
2.2.2. Restriction Endonuclease Digestion of DNA 67
2.2.3. Agarose Gel Electrophoresis of DNA 67
2.2.4. Preparation of DNA Probes 68
2.2.5. Cloning of DNA into Plasmid or Phagemid Vectors 68
2.2.5.1. Vector Preparation 68
2.2.5.2. Filling In Procedure 68

2.2.5.3. Insert Preparation 69
2.2.5.4. Ligation 70
2.2.5.5. Sonication of DNA and Shotgun Cloning into M13mp18 70
2.2.6. Transformation of Bacterial Cells with Recombinant Clones 71
2.2.6.1. Preparation of Hannahan's Competent Cells 71
2.2.6.2. Transfection of Competent Cells 71
2.2.7. Southern Blotting 72
2.2.8. *In Situ* Colony Hybridisation 73
2.2.9. Filter Hybridisation 73
2.2.10. Manual Sequencing of Single-Stranded Plasmid DNA 74
2.2.10.1. Single-Stranded Plasmid DNA Preparation (ssDNA) 74
2.2.10.2. Manual DNA Sequencing 75
2.2.10.3. Acrylamide Gel Electrophoresis for DNA Sequencing 76
2.2.11. Automated Sequencing of Double Stranded Plasmid DNA 76
2.2.11.1. Reactions for Automated Sequencing 76
2.2.11.2. Electrophoresis Using the ABI 377 Automated Sequencer 77
2.2.12. RNA Extraction 78
2.2.13. cDNA Preparation by Reverse Transcriptase 78
2.2.14. Polymerase Chain Reaction (PCR) 79
2.2.15. Site-Directed Mutagenesis 80
2.2.16. Routine Tissue Culture 81
2.2.17. Embryonic Stem Cells 82
2.2.17.1. Routine Culture 82
2.2.17.2. STO Cells 83
2.2.17.3. Preparation of Feeder Cells 83
2.2.17.4. Preparation of Supernatant From CHO 8/24 720 LIF-D(1) 83
2.2.17.5. Transfection of ES Cells 83
2.2.17.6. Karyotyping of ES Cells 84

RESULTS 86

3.1. Identification and Characterisation of Clones Containing Murine Etv6 Exon 5 86
3.1.1. Isolation of Clones Containing Murine Etv6 Exon 5 86
3.1.2. Sequencing Murine Etv6 Exon 5 88
3.2. Generation of the ETV6-CBFA2 Targeting Construct 90

3.2.1. Removal of the 3' Xho I Site....90
3.2.2. Insertion of the Poly-Adenylation Addition Signal....91
3.2.2.1. Removal of the Bam HI site from the pCI Vector and Reintroduction of the Poly(A) Cassette into the ES15Bam/X$^-$ Construct....93
3.2.3. Introduction of the Neomycin Resistance Gene....94
3.2.4. Introduction of the Herpes Simplex Virus Thymidine Kinase Gene....96
3.2.5. Insertion of *CBFA2* cDNA into the ES15Bam/X$^-$poly(A)neo-tk Plasmid....97
3.2.5.1. Mutation of a Xho I Site into Exon 5 of a Human ETV6-CBFA2 cDNA Clone....98
3.2.5.2. Insertion of the Human ETV6-CBFA2 Sequence into the ES15Bam/X$^-$ negative Poly(A)neo-tk Plasmid....100
3.2.6. Sequencing the t(12;21)neo Targeting Construct....101
3.2.7. Preparation of Probes for Detection of Homologous Recombination....102
3.3. Generation of Heterozygous Mutant Etv6/CBFA2 $^{+/-}$ Mice....104
3.3.1. Insertion of the t(12;21)neo Targeting Construct into ES cells by Homologous Recombination....104
3.3.2. Identification of the Etv6-CBFA2 Fusion Transcript....107
3.3.3. Karyotyping Targeted ES Cell Clones....109
3.3.4. Generation of Heterozygous and Homozygous Mutant Etv6/CBFA2 Mice..110
3.3.5. Phenotypic Analysis of Homozygous Mutant Etv6-CBFA2$^{-/-}$ Embryos....116

DISCUSSION....117

4.1. The *Etv6-CBFA2* Fusion Gene Does Not Cause Leukaemia in Chimeric Mice..118
4.2. Heterozygous Expression of the *Etv6-CBFA2* Fusion Gene Does Not Disrupt Normal Development or Give Rise to Leukaemia....119
4.3. Homozygous Expression of Etv6-CBFA2 is Embryonic Lethal....119
4.4. Heterozygousous Expression of Etv6-CBFA2 is Insufficient for Leukaemogenesis....120

CONCLUSIONS....122

APPENDIX I....123

APPENDIX II....130

REFERENCE LIST....131

TABLE OF FIGURES

Figure 1.1. Distribution of Translocation-Generated Fusion Genes Among the Various Morphological Subtypes of AML in Children and Young Adults..... 24

Figure 1.2. Distribution of Translocation-Generated Fusion Genes in the Commonly Recognised Immunologic Subtypes of ALL in Children and Young Adults. 25

Figure 1.3. Distribution of Histologic Subtypes of Non-Hodgkin's Lymphoma in Children and Adults. 26

Figure 1.4. The CBFA2/CBFβ Transcription Factor Complex Binds to the Core Enhancer Sequence and Functions as an Enhancer-Organising Factor to Induce Gene Transcription. 32

Figure 1.5. Structural Organisation of the Full Length CBFA2 cDNA. 34

Figure 1.6. Molecular Consequences of Chromosomal Rearrangements that Target the CBFA2-CBFβ Transcription Factor Complex..... 38

Figure 1.7. Schematic Representation of Full-Length CBFA2, MTG8 and CBFA2-MTG8 Fusion Proteins..... 39

Figure 1.8. The CBFA2-MTG8 Fusion Protein Retains the Ability to Bind to the Core Enhancer Sequence and to Heterodimerise with CBFβ..... 41

Figure 1.9. Diagrammatic Illustration of the Proteins Involved with CBFA2 in the Chimeric Fusion Products Generated by the t(3;21) Translocation..... 44

Figure 1.10. Schematic Representation of CBFβ, MYH11 and CBFβ-MYH11 Proteins.46

Figure 1.11. Structural Organisation of the ETV6 Protein. 49

Figure1.12. Schematic Representation of Full Length ETV6, CBFA2 and ETV6-CBFA2 Fusion Proteins..... 51

Figure 1.13 Schematic Representation of ETV6, PDGFRβ and ETV6-PDGFRβ Fusion Proteins. 53

Figure 1.14. Schematic Diagram Illustrating the Stage at which Transcription Factors Influence Haematopoietic Development..... 58

Figure 3.1. Restriction Map of λES15. 87

Figure 3.2. Restriction Map of the 6 kb ES15Bam Subclone. 88

Figure 3.3. Sequence Analysis of the Murine ES15Bam Clone and Comparison with the Published Human and Murine Sequences of *ETV6* Exon 5..... 89
Figure 3.4. Sequence of the Area Surrounding the 3' Xho I Site and Oligonucleotide Primers Designed to Replace the Xho I site with Cla I. 90
Figure 3.5. Linear Map of the Relevant Region of the 4006 bp pCI Vector. 91
Figure 3.6. Schematic Explanation of the Retention of Bam HI and Eco RI Restriction Sites After Digestion and Filling in with T4 DNA Polymerase. 92
Figure 3.7. Sequence of pCI Plasmid Surrounding the 3' Bam HI Site..... 93
Figure 3.8. Schematic Representation ES15Bam/X$^-$Poly(A) Construct. 94
Figure 3.9. Linear Map of the Relevant Region of the pMCI Neo Vector. 95
Figure 3.10. Schematic Representation of the ES15Bam/X$^-$poly(A)neo Cassette..... 96
Figure 3.11. Linear Map of the pTK (ES) Construct. 96
Figure 3.12. Schematic Representation of the ES15bam/X$^-$poly(A)neo cassette in Bluescript-tk Vector. 97
Figure 3.13. Diagrammatic Representation of the 2.5 kb ETV6-CBFA2R Clone. 98
Figure 3.14. Sequence of Murine *Etv6* Surrounding the Internal Xho I Site Compared to the Same Area in the ETV6-CBFA2R Clone. 99
Figure 3.15. Diagrammatic Representation of the ETV6-CBFA2R/X$^+$ Clone..... 100
Figure 3.16. Diagrammatic Representation of the t(12;21)neo Targeting Construct. ... 101
Figure 3.17. Restriction Map of the *Etv6* Gene in the Proximity of Exon 5 and the Predicted Structure of the Targeted Etv6 Locus Following a Homologous Recombination Event. 103
Figure 3.18. Screening for *Etv6* Targeted ES Cell Clones..... 105
Figure 3.19. Confirmation of Targeted Clones. 106
Figure 3.20. Identification of the ETV6 and ETV6-CBFA2 fusion products..... 108
Figure 3.21. G-Banded Karyotype of ES Clone 1F1. 109
Figure 3.22. Confirmation of Germ-Line Transmission of the Etv6-CBFA2 Mutation. 110
Figure 3.23. Genotyping dE10.5 and dE11.5 Embryos 115
Figure 3.23. Phenotype of Embryos Resulting From Crossing Etv6-CBFA2 Heterozygous Mice. 116

TABLE OF TABLES

Table 1.1A. Gene Enhancements. 17

Table 1.1B. Gene Fusions. 19

Table 1.2. Features of the Three Members of the CBFα Transcription Factor Family. .. 30

Table 3.1. Electroporation of ES cells with Etv6 Targeting Vector..... 104

Table 3.2. Details of mice Generated by the Indicated Matings 111

Table 3.3. Genotype of Embryos Analysed at dE10.5 and dE11.5. 114

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ABBREVIATIONS

ABL	Abelson
ABS	ATP-binding site
AGM	Aorta, gonad, mesonephros
AL	Acute leukaemia
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloblastic leukaemia
APL	Acute promyelocytic leukaemia
ARG	Abelson-related gene
AUL	Acute undifferentiated leukaemia
BC	Blast crisis
BCR	Breakpoint cluster region
Bgb	Big brother
bHLH	Basic region helix-loop-helix
BL	Burkitt's lymphoma
Bro	Brother
bZIP	Basic region leucine-zipper
c-ALL	Common acute lymphoblastic leukaemia
CBF	Core-binding factor
CBP	Creb Binding protein
CCD	Cleidocranial dysplasia
CL	Centrocytic lymphoma
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CMML	Chronic myelo-monocytic leukaemia
CSF	Colony stimulating factor
DDW	Double distilled water
DEPC	Diethyl pyrocarbonate
DLCL	Diffuse large cell lymphoma
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleaic acid

DNAase	Deoxyribonuclease
dNTPs	Deoxyribonucleoside triphosphates
DTT	Dithiothreitol
EAP	Epstein-Barr virus RNA-associated protein
EDTA	Disodium ethylenediaminetetra acetate
ENU	N-ethyl-N-nitrosourea
ES	Embryonic stem
ETO	Eight twenty one
ETS	E26-transformation specific
ETV	ETS-type variant
FAB	French, American, British
FIAU	1-(2-deoxy, 2-fluoro-β-Darabinofuanosil)-5-idouracil
FISH	Fluorescent *in situ* hybridisation
FL	Follicular lymphoma
GM-CSF	Granulocyte-macrophage colony stimulating factor
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HCL	Hairy cell leukaemia
HHR	Hydrophobic heptad repeat
HSC	Haematopoietic stem cell
Hyg	Hygromycin
Ig	Immunoglobulin
IL-3	Interleukin-3
JAK	Janus kinase
LIM	Lin-11 insulin-1 mec-3
LPL	Lymphoplasmacytoid lymphoma
MDS	Myelodysplastic syndrome
MPD	Myeloproliferative disease
MPO	Myeloperoxidase
MYB	Myeloblastosis
MYC	Myelocytomatosis
MYH	Myosin heavy chain
MTG	Myeloid translocation gene

N-CoR	Nuclear receptor corepressor
Neo	Neomycin
NHR	Nervy homology region
NHL	Non-Hogdgkin's lymphoma
NM	Nuclear matrix attachment
NTRK3	Neurotrophin-3 receptor tyrosine kinase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF(R)	Platelet derived growth factor (receptor)
PEG	Polyethylene glycol
Ph^1	Philadelphia chromosome
PLL	Prolymphocytic leukaemia
PNT	Pointed
PST	Proline-serine-threonine-rich
RAR	Retinoic acid receptor
RHD	Runt homology domain
RNA	Ribonucleic acid
RNAase	Ribonuclease
SDS	Sodium dodecyl sulphate
SMMHC	Smooth muscle myosin heavy chain
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
STAT	Signal transducer and activator of transcription
TA	Transcriptional activation
TAF	Transcriptional-activating factor
t-AML	Therapy related acute myeloblastic leukaemia
TBE	Tris-borate/EDTA
TCR	T-cell receptor
TEL	Translocated ETS leukaemia
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF	Transforming growth factor
TK	Thymidine kinase

INTRODUCTION

The t(12;21) translocation results in the fusion of the dimerisation domain of the *ETV6* gene, an Ets-like transcription factor situated on chromosome 12 band p13, to the DNA-binding domain of the transcription factor *CBFA2* on chromosome 21 band q22.[1] The translocation is seen in approximately 25% of cases of childhood ALL, making the *ETV6-CBFA2* fusion the most common genetic abnormality in lymphoid leukaemias.[2] The principal aim of this thesis is to investigate the role of the *ETV6-CBFA2* fusion gene in leukaemogenesis.

This first chapter gives an overview of some of the best-characterised chromosomal abnormalities in hematological malignancies and describes the relevant associated molecular changes. The t(12;21) translocation involving the *ETV6* and *CBFA2* genes is discussed in more detail, as it is most relevant to the subject of this thesis. Finally, some of the experimental models used to investigate the role of chromosomal translocations in leukaemogenesis are discussed.

1.1. Haematological Malignancy

The haematological malignancies are usually divided into two broad groups the leukaemias and the lymphomas.

1.1.1. Leukaemia

The leukaemias are a group of malignant disorders characterised by the accumulation of white cell precursors in the bone marrow and peripheral blood. These abnormal cells may cause a raised circulating white cell count, bone marrow failure and organ infiltration.

They are sub-divided into acute and chronic leukaemia. Acute leukaemia (associated with over 50% of immature blast cells in the bone marrow at clinical presentation), is further divided into acute myeloblastic leukaemia (AML) and acute lymphoblastic leukaemia (ALL) on the basis of morphology, cytochemistry, immunophenotype, chromosome and gene rearrangement studies. AML and ALL are

further subdivided on morphological basis, according to the French-American-British (FAB) scheme.[3]

The chronic leukaemias comprise two main types, chronic myeloid leukaemia (CML) and chronic lymphocytic leukaemia (CLL). Other chronic types include hairy cell leukaemia (HCL), prolymphocytic leukaemia (PLL) and various leukaemia/lymphoma syndromes. In addition, there are a variety of myelodysplastic syndromes (MDS), some of which are regarded as chronic forms of leukaemia and others as "pre-leukaemia".

1.1.2. Malignant Lymphoma

This group of diseases is divided into Hodgkin's disease and non-Hodgkin's lymphoma (NHL). In both, there is replacement of lymphoid structure by collections of abnormal cells, Hodgkin's disease being characterised by the presence of Reed-Sternberg cells and NHL by diffuse or nodular collections of abnormal lymphocytes or, rarely, histiocytes.

Much is now known about the genetic and molecular changes in haematopoietic cells associated with malignant transformation, and general aspects of these changes are now discussed.

1.2. Chromosomal Changes and Malignancy

An important concept in the modelling of carcinogenesis is the knowledge that cancer cells contain genetic material, which is organised and/or functions differently from normal cells. Deletions (a loss of genetic material), translocations (the exchange of genetic material between two chromosomes or between distinct regions on the same chromosome), inversions (intrachromosomal relocation of genetic material), and point mutations (single base pair changes in coding or non-coding regions) disrupt gene function and lead to cancer.[4-6] By far the most common genetic abnormalities thus far identified in haematological malignancies involve chromosomal translocations.

1.2.1. Chromosomal Translocations

Molecular analysis of translocations has led to both the identification of a number of novel genes and further understanding of the mechanisms leading to chromosomal

abnormalities. While some of the genes involved were known oncogenes, (such as the cellular homologue of the Abelson murine leukaemia virus, *c-ABL*, or the myelocytomatosis gene, *cMYC*), many were previously unknown genes that have subsequently been shown to play important roles in cell growth and development. The majority of the genes involved in chromosomal translocations are transcription factors, of which four major types can be identified on the basis of recurring structural elements within their DNA- and protein-binding domains. These are: (1) basic region/helix-loop-helix (bHLH), (2) basic region leucine-zipper (bZIP), (3) zinc finger, and (4) homeodomain. Other functional motifs include A-T hook, Ets-like, Runt homology and cysteine-rich (LIM) domains. [6-8]

There are two principle types of translocations: (1) translocations involving an antigen receptor site and (2) translocations involving a non-antigen receptor site. In the former type, the genes encoding for T-cell receptor (TCR) or immunoglobulin (Ig) proteins become adjacent, via the translocation, to a region frequently containing a proto-oncogene. This event results in activation or enhancement of the oncogene expression.

In the latter type, the chromosomal breaks disrupt the organisation of genes on the two chromosomes involved, creating a fusion gene encoding a chimeric protein. None of the two partner genes is an antigen receptor gene. Tables 1.1A and 1.1B attempt to provide a comprehensive summary of translocations and molecular details identified in haematological malignancies. The translocations have been listed according to primary criteria; i.e. whether they produce a gene enhancement (Table 1.1A) or gene fusion (Table 1.B) as briefly described above.

Table 1.1A. Gene Enhancements.

Protein Family	Translocation	Affected Gene	Rearranging Gene	Disease	Ref.
Basic helix-loop-helix	t(8;14)(q24;q32)	MYC	IgH	BL	9; 10
	t(2;8)(p12;q24)	MYC	IgK	B-ALL	9; 10
	t(8;22)(q24;q11)	MYC	IgL	BL	9; 10
	t(8;14)(q24;q11)	MYC	TCRα	T-ALL	11; 12
	t(8;12)(q24;q22)	MYC/BTG		B-CLL/ALL	13; 14
	t(7;19)(p35;p13)	LYL1	TCRβ	T-ALL	15
	t(1;14)(p32;q11)	TAL1	TCRα/δ	T-ALL	16; 17
	t(7;9)(q34;q32)	TAL2	TCRβ	T-ALL	18
Cysteine-rich (LIM)	t(11;14)(p15;q11)	LMO1	TCRδ	T-ALL	19
	t(11;14)(p13;q11)	LMO2	TCRα/δ	T-ALL	20; 21
	t(7;11)(q35;p13)	LMO2	TCRβ	T-ALL	22
Homeobox	t(10;14)(q24;q11)	HOX11	TCRα	T-ALL	23; 24
	t(7;10)(q35;q24)	HOX11	TCRβ	T-ALL	25
	t(9;14)(p13;q32)	PAX5	IgH	LPL	26

Table 1.1A. (cont.).

Protein Family	Translocation	Affected Gene	Rearranging Gene	Disease	Ref.
Zinc-finger	t(3;14)(q27;q32)	BCL-6	IgH	NHL/DLCL	27; 28
	t(3;4)(q27;p11)	BCL-6	TTF	NHL	29; 30
	t(3; v)(q26; v)	EVI1		AML	31; 32
Others	t(14;18)(q32;q21)	BCL2	IgH	FL	33-35
IκB homology	t(14;19)(q32;q13.1)	BCL3	IgH	B-CLL	36; 37
	inv14/t(14;14)(q11;q32)	TCL1	TCRα	T-CLL	38
REL homology	t(10;14)(q24;q32)	LYT-10	IgH	NHL	39
EGF cysteine repeats	t(7;9)(q34;q34)	TAN-1	TCRβ	T-ALL	40
Tyrosine kinase	t(1;7)(p34;q34)	LCK	TCRβ	T-ALL	41; 42
G1 cyclin	t(11;14)(q13;q32)	Cyclin D1	IgH	B-CLL/CL	43; 44
Growth factor	t(5;14)(q31;q32)	IL-3	IGH	Pre-B-ALL	45; 46
Unknown	t(X;14)(q28;q11)	C6.1B	TCRα	T-PLL	47; 48

Table 1.1B. Gene Fusions.

Translocation	Affected Genes	Protein Domains	Disease	Ref.
inv 14(q11;q32)	TCRα VH	TCRCα Ig VH	NHL	49; 50
t(17;19)(q22;p13)	HLF E2A	BZIP AD-bHLH	Pro-B ALL	51; 52
t(1;19)(q23;p13)	E2A PBX1	AD-bHLH Homeobox	Pre-B ALL	53-55
t(15;17)(q21;q21)	PML RARα	Zinc-finger Retinoic acid receptor	APL	56-58
t(11;17)(q23;q21)	PLZF RARα	Zinc-finger Retinoic acid receptor	APL	59
t(5;17)(q32;q12)	NPM RARα	Nucleophosmin Retinoic acid receptor	APL	60
t(4;11)(q21;q23)	MLL AF4	A-T hook/Zn finger Ser-Pro rich	Pre-B ALL	61-63
t(6;11)(q27;q23)	MLL AF6	A-T hook/Zn finger Ras binding	AML	64
t(9;11)(p21;q23)	MLL AF9	A-T hook/Zn finger Ser-Pro rich	ALL AML	65
t(10;11)(p12;q23)	MLL AF10	A-T hook/Zn finger Leucine-zipper	AML	66

Table 1.1B. (cont. 1).

Translocation	Affected Genes	Protein Domains	Disease	Ref.
t(11;17)(q23;q21)	MLL AF17	A-T hook/Zn finger Leucine-zipper	AL	67
t(1;11)(p32;q23)	MLL AF1P	A-T hook/Zn finger Eps 15 homologue	AML	68
t(1;11)(q21;q23)	MLL AF1Q	A-T hook/Zn finger Unknown	AML	69
t(X;11)(q13;q23)	MLL AFX1	A-T hook/Zn finger Forkhead DNA-binding	ALL	70; 71
t(11;19)(q23;p13.3)	MLL ENL	A-T hook/Zn finger Ser-Pro rich	ALL/AML	65; 72; 73
t(11;19)(q23;p13.1)	MLL ELL	A-T hook/Zn finger Lysine rich	AML	74; 75
t(11;16)(q23;p13)	MLL CBP	A-T hook/Zn finger Transcriptional coactivator	MDS	76
t(8;16)(p11;p13)	MOZ CBP	Transcriptional coactivator Transcriptional coactivator	AML	77
inv(8)(p11;q13)	MOZ TIF2	Transcriptional coactivator Transcriptional coactivator	AML	78; 79
t(9;22)(q34;q11)	ABL BCR	Tyrosine kinase Serine-threonine kinase	CML ALL	80-82 83-85
t(9;12)(q34;p13)	ABL ETV6	Tyrosine kinase ETS-related DNA binding	ALL AML	86 87-89

Table 1.1B. (cont. 2).

Translocation	Affected Genes	Protein Domains	Disease	Ref.
t(1;12)(q25;p13)	ARG ETV6	Tyrosine kinase ETS-related DNA binding	AML	90
t(1;12)(q21;p13)	ARNT ETV6	Transcriptional coactivator ETS-related DNA binding	AML	91
t(5;12)(q33;p13)	PDGFRβ ETV6	Receptor kinase ETS-related DNA binding	CMML ALL	92 93
t(9;12)(p24;p13)	JAK2 ETV6	Tyrosine kinase ETS-related DNA binding	ALL/CML	94
t(12;15)(p13;q25)	ETV6 TRKC	ETS-related DNA binding Tyrosine kinase receptor	AML	95
t(12;22)(p13;q11)	ETV6 MN1	ETS-related DNA binding Unknown	MDS/AML	96
t(3;12)(q26;p13)	EVI1 ETV6	Zn finger ETS-related DNA binding	MDS CML	97; 98 97
t(4;12)(q11-13;p13)	BTL ETV6	Brx homology ETS-related DNA binding	AML M0	99
t(12;13)(p13;q12)	ETV6 CDX2	ETS-related DNA binding Homeobox	AML	100
t(5;12)(q31;p13)	ASC2 ETV6	Acyl CoA synthetase ETS-related DNA binding	MDS/AML	101
t(12;21)(p13;q22)	ETV6 CBFA2	ETS-related DNA binding Runt DNA binding	cALL	1; 102

Table 1.1B. (cont. 3).

Translocation	Affected Genes	Protein Domains	Disease	Ref.
t(8;21)(q22;q22)	MTG8 CBFA2	Zn finger Runt DNA binding	AML	103; 104
t(16;21)(q24;q22)	MTG16 CBFA2	Zn finger Runt DNA binding	BC MDS t-AML	105
t(3;21)(q26;q22)	EVI1 CBFA2	Zn finger Runt DNA binding	CML t-AML, MDS	106
t(3;21)(q26;q22)	EAP CBFA2	Ribosomal protein Runt DNA binding	MDS	107
inv(16)(p13;q22) t(16;16)(p13;q22)	MYH11 (p13) CBFβ (q22)	SMMHC Binds to CBFA2	AML	108
t(16;21)(p11q22)	FUS ERG	RNA binding protein ETS-related DNA binding	AML	109; 110
t(6;9)(p23;q24)	DEK CAN	Unknown Nucleoporin	AML	111; 112
t(9;9)(q34;p34)	SET CAN	Unknown Nucleoporin	AUL	113
t(1;11)(q23;p15)	PMX1 NUP98	Homeodomain Nucleoporin	AML	114
t(4;11)(q21;p15)	RAP1GDS1 NUP98	Guanine exchange factor Nucleoporin	T-ALL	116
t(7;11)(p15;p15)	HOXA9 NUP98	Homeodomain Nucleoporin	AML	117; 118

Table 1.1B. (cont. 4).

Translocation	Affected Genes	Protein Domains	Disease	Ref.
inv(11)(p15;q22}	DDX10	RNA helicase	AML	119
	NUP98	Nucleoporin		
t(2;11)(q31;p15)	HOXD13	Homeodomain	AML	115
	NUP98	Nucleoporin		
t(1;2)(q25;p23)	TPM3	Tropomyosin	NHL	120
	ALK	Tyrosine kinase receptor		
t(2;5)(p23;q25)	ALK	Tyrosine kinase receptor	NHL	121
	NPM	Nucleolar shuttle protein		
t((3;5)(q35;q35)	MLF1	Unknown	MDS	122
	NPM	Nucleolar shuttle protein	AML	
t(8;13)(p11;q12)	FGFR1	Tyrosine kinase receptor	MPD	123; 124
	FIM	Zn finger		
t(6;8)(q27;p11)	FOP	Leucine rich	MPD	125
	FGFR1	Tyrosine kinase receptor		
t(10;11)(p13;q14)	AF10	Unknown	AML	126
	CALM	Clathrin assembly		
inv(2;2)(p13;p11.2-14)	REL	DNA binding activator	NHL	127
	NRG	Unknown		
t(4;16)(q26;p13)	IL-2	Growth factor	NHL	128
	BCM	Unknown		

Although many chromosomal translocations have now been described, a relative few appear to be involved in the majority of haematopoietic malignancies. Furthermore, there are significant differences in the classes of genes involved in chromosomal translocations associated with different malignancies. Figures 1.1, 1.2 and 1.3 display graphically the distribution of the more common chromosomal translocations found in adult and childhood leukaemias and lymphomas.

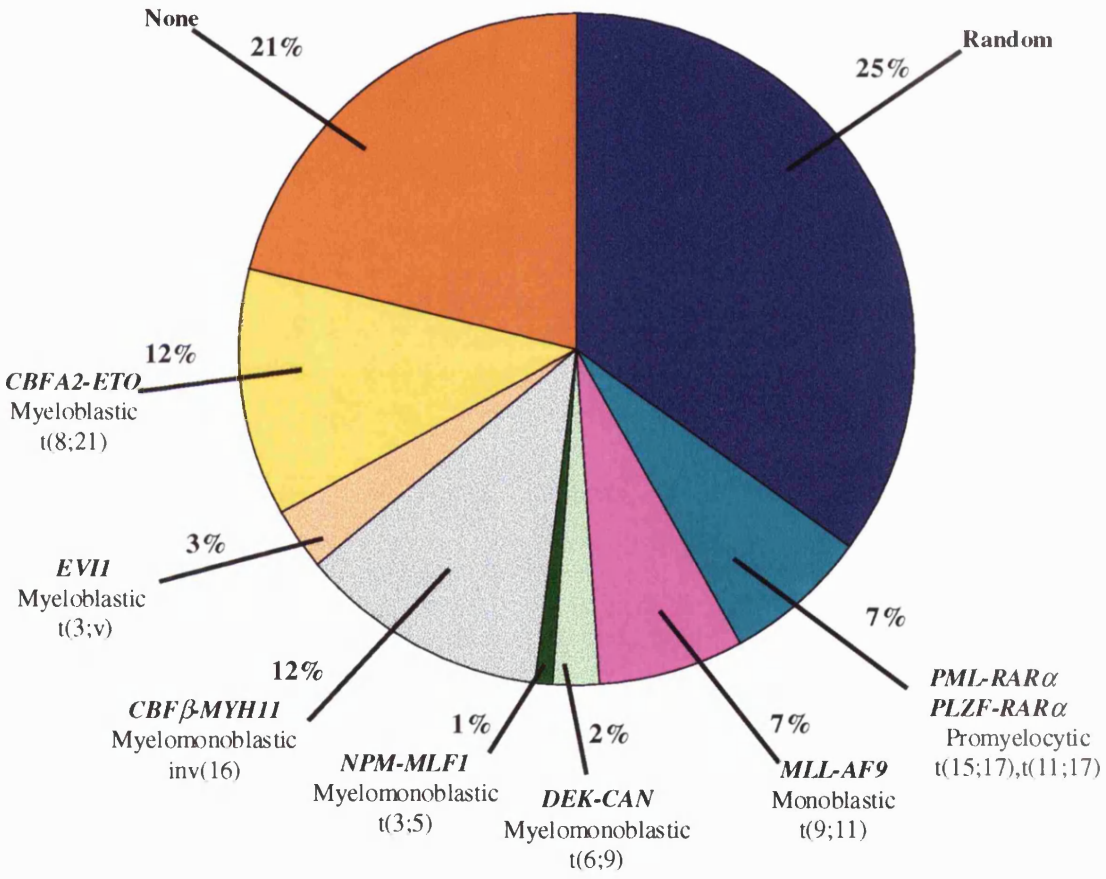


Figure 1.1. Distribution of Translocation-Generated Fusion Genes Among the Various Morphological Subtypes of AML in Children and Young Adults.

The section labelled "random" refers to sporadic rearrangements that have so far only been observed in leukaemic cells from single cases. Adapted from ref.[129]

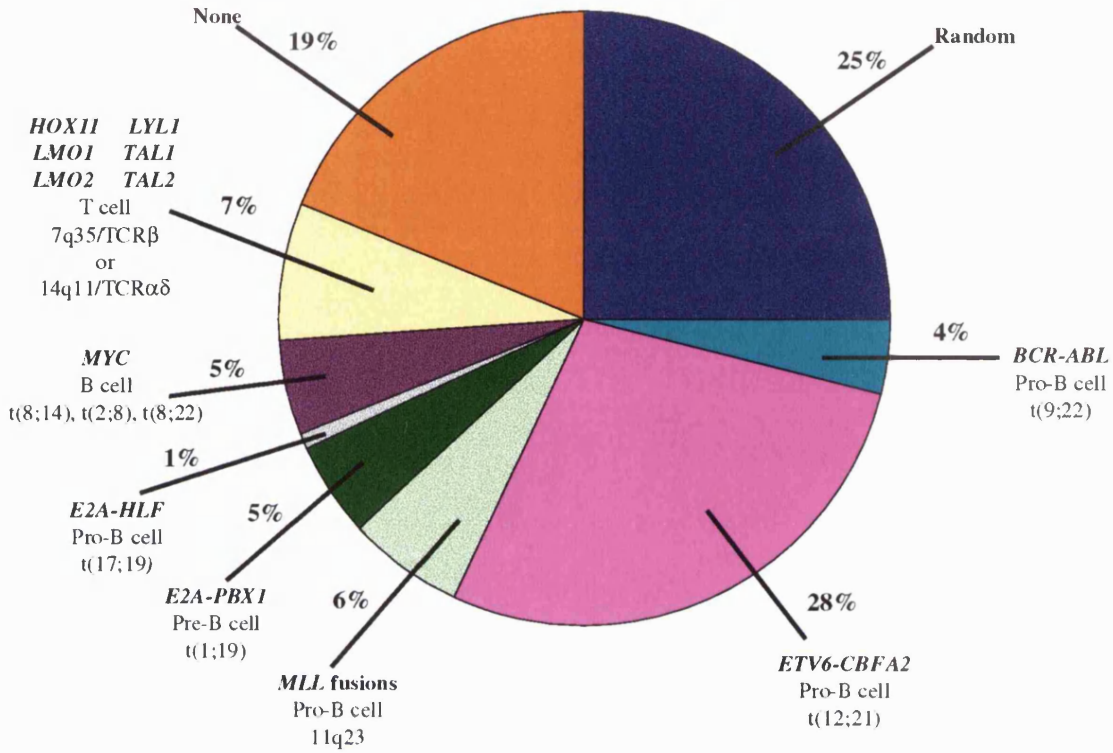


Figure 1.2. Distribution of Translocation-Generated Fusion Genes in the Commonly Recognised Immunologic Subtypes of ALL in Children and Young Adults.

The section labelled "random" refers to sporadic rearrangements that have so far only been observed in leukaemic cells from single cases. Adapted from ref.[129]

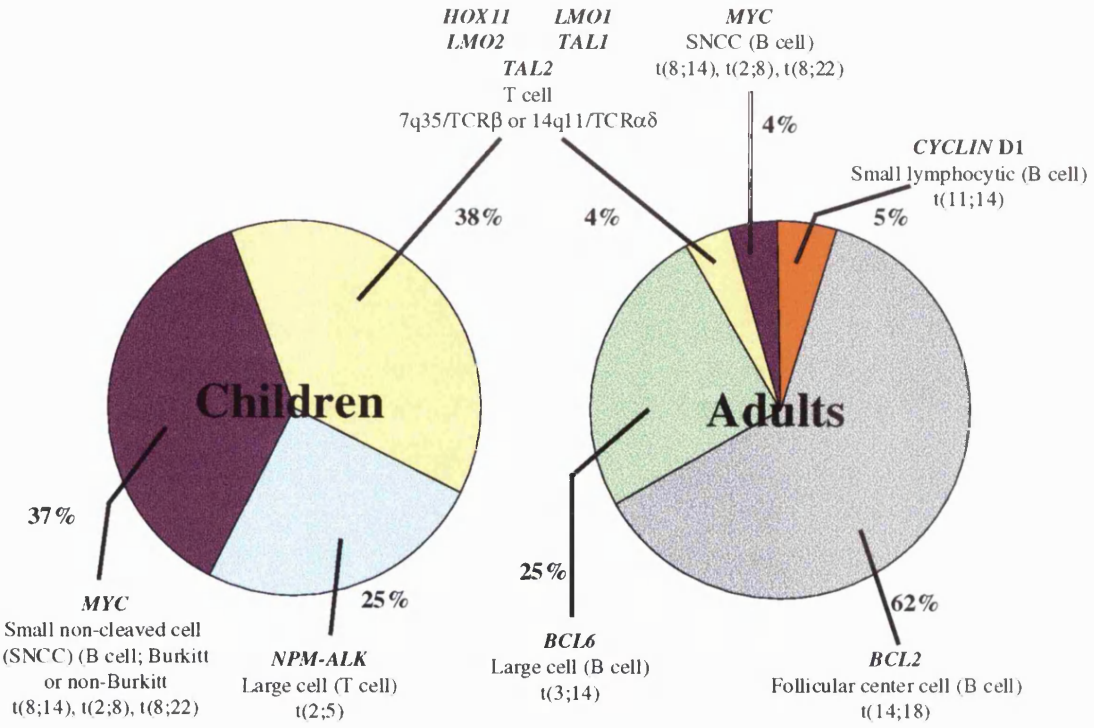


Figure 1.3. Distribution of Histologic Subtypes of Non-Hodgkin's Lymphoma in Children and Adults.

Chromosomal translocations and affected genes that occur in a significant fraction (but not all) of the cases within each subtype are shown. Adapted from ref.[130]

I will briefly describe below two chromosomal translocations that historically have been instrumental in the understanding of molecular changes associated with leukaemogenesis, to illustrate the consequences of either a gene enhancement or gene fusion type of rearrangement

1.2.1.1. Gene Enhancement Via Antigen Receptor Gene Activation: The Burkitt's Lymphoma Model

The Immunoglobulin (Ig) and T cell receptor (TCR) gene loci are involved in a variety of chromosomal translocations. However, this is almost exclusively seen in B- and T-cells where Ig and TCR genes are rearranged to generate functional antigen receptor molecules. This process, complex and only partially understood, can lead to inter- or intra-chromosomal translocations or inversions and is most commonly associated with erroneous V-D-J recombination.[131] The Ig genes are distributed as follows: the heavy chain (IgH) encoding segments are situated on chromosome 14 band q32, the light chain κ and λ gene segments on chromosome 2p12 and 2q22, respectively. In virtually all cases of Burkitt's lymphoma (BL) an erroneous recombination between the *cMYC* gene (on chromosome 8q24) and one of the three Ig loci results in the *cMYC* gene coming under the control of the Ig sequences. The sporadic or endemic form of the disease displays the major type of the t(8;14)(q24;q32). In this form a chromosomal region upstream of the *cMYC* gene is joined, via the translocation, to the joining or variable region of the IgH genes.[10; 132] Conversely, in the variant type of BL the downstream portion of the *cMYC* gene can be translocated to the k or λ Ig light chain locus in t(2;8)(p12;q24) or t(8;22)(q24;q22), respectively. [133; 134] Due to the proximity of the *cMYC* gene to the enhancer sequences (downstream to the JH segments) or the promoter sequences (upstream to the variable segments) of the Ig genes, the *cMYC* gene becomes transcriptionally deregulated with subsequent inappropriate expression.

1.2.1.2. Gene Fusion

By far the most frequently described chromosomal rearrangements lead to the fusion of genes into functional chimeras. Such fusions give rise to novel proteins capable of interacting with DNA and other regulatory elements, in ways that disrupt normal cellular control mechanisms.

A typical example of such a translocation is the BCR/ABL fusion resulting from the t(9;22)(q34;q22) translocation in Philadelphia positive (Ph^1) leukaemias,[80] but other translocations frequently seen in lymphoid leukaemias will be briefly discussed.

1.2.1.2.1. Philadelphia Positive Leukaemia

Three alternate forms of the Ph^1 chromosome translocation are found in CML, ALL, and granulocytic CML, respectively. They result from the joining of different exons of the breakpoint cluster region (*BCR*) gene on chromosome 22q22 to exon 2 of the Abelson (*ABL*) gene located on chromosome 9q34.[135] These events result in alternative chimeric fusion proteins, p210 BCR-ABL, p190 BCR-ABL, and p230 BCR-ABL, in which exon 1 (in Ph^1 ALL predominantly, and rarely, in CML), exon 13 or 14 (in CML or, more infrequently, in ALL) and exon 19 (in granulocytic CML) of the *BCR* gene can be commonly translocated to intron 2 of the *ABL* gene (reviewed in [136]). The incidence of the t(9;22) is age related as it has been described in less than 5% of childhood ALL, but in over 25% of adult ALL.

1.2.1.2.2. The t(12;21) Translocation in Paediatric ALL

Until relatively recently, the most common translocation identified in paediatric ALL, detected in 5-10% of patients, was the t(1;19)(q23;p13) translocation leading to the fusion of the E2A and PBX1 genes[137] and the t(4;11)(q21;q23) translocation, detected in 2% of children [138] and up to 30% of infants with ALL,[139] leading to the fusion of the mixed-lineage leukaemia (MLL) and AF4 genes. An even more common translocation in childhood ALL has recently been identified in the t(12;21)(p13;q22) translocation. This is rarely detected by routine karyotyping because the telomeric segments of 12p and 21q appear similar in banded metaphase preparations. However, this rearrangement is apparent by fluorescence in situ hybridisation (FISH) in approximately 25% of children with ALL, making t(12;21)(p13;q22) the most common genetic abnormality in the lymphoid leukaemias.[2] Like t(9;22), the t(12;21) translocation is also age restricted as it is rarely detected in adult ALL (less than 3%). As many other translocations, it has a lineage restricted incidence, since it has almost exclusively been described in B-cell lymphoid malignancies.

The t(12;21)(p13;q22) breakpoint has been cloned and shown to fuse the Ets-type variant 6 (*ETV6*, previously known as *TEL* [translocated ETS leukaemia]) gene on 12p13 to the core binding factor A2 (*CBFA2* also known as *AML1*) gene on 21q22.[1; 102] The *ETV6* gene was initially identified by cloning a leukaemia-associated translocation breakpoint, the t(5;12)(q33;p13), described in chronic myelomonocytic leukaemia (CMML).[92]

CBFA2 is the DNA-binding component of the core-binding factor (CBF) transcription complex and was first identified through its fusion to MTG8 (Myeloid Translocation Gene on chromosome 8) in the t(8;21)(q22;q22) translocation which occurs in ~40% of cases of AML (M2). [104] Subsequently the CBF complex has been shown to be the most frequent target of chromosomal translocations in the human leukaemias, in that, part of the complex is expressed as an oncogenic chimera in as many as one-third of both ALL and AML cases, the most common of which are inv (16), t(8;21), and t(12;21).

As the t(12;21) translocation is the focus of this study, the next sections will describe in detail the genes involved. The products of the genes comprising the CBF complex and the *ETV6* gene will be discussed along with their involvement in chromosomal translocations associated with other haematological malignancies. Finally, the mechanisms by which these translocations may cause malignant transformation will be discussed.

1.3. The Core Binding Factor Complex.

Core binding factor (CBF) is a DNA-binding transcription factor complex composed of α and β subunits.[140] The CBFα subunit is the DNA binding element of the complex and is capable of binding DNA *in vitro* in the absence of its partner protein, CBFβ.[141-144] CBFβ stabilises the binding of CBFα to DNA without contacting DNA itself. [143-145] Three mammalian α subunits have been identified, termed CBFA1 (AML3, PEBP2αA), CBFA2 (AML1, PEBP2αB), and CBFA3 (AML2, PEBP2αC),[104; 142; 146; 147] whereas only a single β subunit, CBFβ (PEBP2β) is present in mammals.[141] All members of the CBFα family are structurally very similar and only appear to differ in the specificity of tissue expression (Table 1.2). All three CBFα subunits contain a highly conserved 118 amino acid domain that is homologous to the *Drosophila* pair-rule protein Runt (hence its designation as the runt homology domain or RHD).[148; 149] The RHD is the DNA-binding domain of the CBFα protein and also contains the heterodimerisation domain for the CBFβ sub-unit.[142; 143; 149]

	CBFA1	CBFA2	CBFA3
Chromosomal Location	Chromosome 6p21	Chromosome 21q22	Chromosome 1p36
Conserved Domains	RHD, PST, ABS, Groucho binding	RHD, PST, ABS, Groucho binding	RHD, PST, ABS, Groucho binding
Dimerisation Partner	CBFβ	CBFβ	CBFβ
Cellular Localisation	Nuclear	Nuclear	Nuclear
Expression Pattern	Osteoblast lineage	Highest in haematopoietic lineage	Predominantly in haematopoietic lineage

Table 1.2. Features of the Three Members of the CBF Transcription Factor Family.

Abbreviations are as follows: RHD, runt homology domain; PST, proline-serine-threonine rich domain; ABS, ATP-binding site.

The *Drosophila runt* gene participates in several developmental processes, including sex determination, segmentation and neurogenesis (Runt).[150-152] A *Drosophila* homologue of the *runt* gene called *lozenge* is involved in the pathway that specifies photoreceptor cell identity during eye development[153] and has recently been shown to be essential for the development of early haematopoietic (crystal) cells during embryonic and larval haematopoiesis.[154] Recently, a *Caenorhabditis elegans* homologue of the mammalian *CBFα* gene, named *run*, has been isolated. The *run* gene encodes a 301 amino acid protein with a highly conserved RHD.[155] In humans, the three CBFα subunits have been shown to play critical roles in both normal developmental processes and disease. Much of the data concerning the structure and function of the CBFα subunit has been ascertained from the CBFA2 protein, as this was the first to be characterised. I will therefore describe in detail the CBFA2 subunit with particular reference to its structure,

function and role in disease before discussing the other two members of the family and finally the CBFβ subunit.

1.3.1. CBFA2

The *CBFA2* gene was first identified in 1991 through its involvement in the t(8;21)(q22;q22)[104] and was subsequently found to belong to a new family of transcription factors. During development, high levels of CBFA2 can be detected in haematopoietic stem cells (HSC), endothelial cells of the aorta, gonad, mesonephros (AGM) region, chondrogenic centres, olfactory and gustatory mucosa, and neural ganglion cells.[156-158] After organogenesis, CBFA2 expression is primarily restricted to cells of the haematopoietic lineage.[157]

The sequence element recognised by CBFA2 is TGTGGT. This is an enhancer core motif that serves as a regulatory element in several viral enhancers, as well as genes whose products are involved in the regulation of haematopoiesis, such as interleukin-3 (IL-3), granulocyte-macrophage-colony stimulating factor (GM-CSF), colony stimulating factor 1 (CSF-1), myeloperoxidase (MPO), and the T-cell receptors.[143; 144; 159-165] However, although binding of CBFA2 to the core enhancer sequence is important for expression of these genes, adjacent binding sites for lineage-restricted transcription factors, such as c-MYB, C/EBP-α, and ETS family members are also important (Figure 1.4).[160; 161; 166-168] Thus, CBFA2/CBFβ may function as a transcriptional organiser that recruits specific factors into a complex that stimulates lineage specific transcription.[169] This hypothesis is supported by the finding that CBFA2 synergistically activates transcription of the *TCRβ* and *TCRα* enhancers with Ets1,[164; 170] the *NP-3* promoter with C/EBP-α,[171] and the *CSF-1R* promoter with both C/EPB-α and PU.1.[172] These functions appear to involve a direct physical interaction between CBFA2 and the co-operating transcription factor, resulting in both enhanced DNA binding of each factor and the generation of an activation surface which facilitates interactions with co-activators and the basal transcriptional machinery (Figure 1.4).[169]

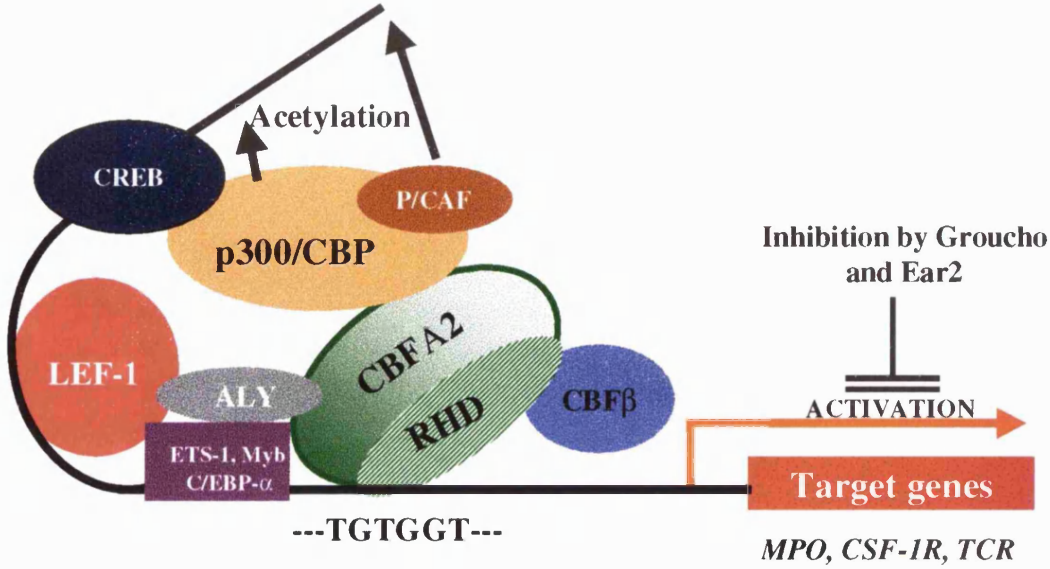


Figure 1.4. The CBFA2/CBFβ Transcription Factor Complex Binds to the Core Enhancer Sequence and Functions as an Enhancer-Organising Factor to Induce Gene Transcription.

Other proteins in this complex differ according to the particular promoter or enhancer involved. They include the transcription factors Ets-1 or Ets family members, C/EBP–α, the DNA-bending protein LEF-1, which interacts with CBFA2 through an adapter protein called ALY and the transcriptional co-activators p300/CBP. p300/CBP recruits other basal transcription factors such as CREB and also binds to the histone acetyltransferase, P/CAF. Among the genes, whose transcription is regulated by CBFA2, we find myeloperoxidase (MPO), the receptor for colony-stimulating factor 1 (CSF-1R) and the T-cell antigen receptor (TCR). Binding of the AML/CBFβ complex to DNA normally leads to transcriptional activation; however, when it is complexed with either Groucho or Ear2 protein, its activity is converted to that of a transcriptional repressor. Adapted from refs.[174; 175]

Although lineage specific transcription usually involves the recruitment of specific factors which can co-operatively bind DNA, transcriptional synergy between

CBFA2 and c-MYB appears to occur without co-operative binding to the *TCRδ* enhancer or the myeloperoxidase promoter.[161] However, this transcriptional synergy again appears to result from interaction of these transcription factors with components of the basal transcriptional machinery.

Additional sequence-specific DNA-binding proteins such as LEF-1 also influence the activity of CBFA2 [170]. LEF-1 facilitates interactions between CBFA2 and adjacently bound co-activators by binding to the minor groove of DNA and inducing a bend in the enhancer sequence. The ubiquitous co-activator ALY directly binds to both CBFA2 and LEF-1, thereby stabilising their juxtaposition (Figure 1.4).[173]

CBFA2-mediated transcriptional activation has also been shown to involve binding the transcriptional co-activators p300 or Creb binding protein (CBP) to the transcriptional activation domain of CBFA2 (Figure 1.4).[175] These co-activators may bind other basal transcription factors such as CREB. P300/CBP have intrinsic histone acetyltransferase (HAT) activity, and also bind to a second HAT, P/CAF.[176; 177] Together, these HATs induce the acetylation of lysine residues in chromatin-associated histones, resulting in a change in a chromatin structure that leads to enhanced transcription (Figure 1.4).[178; 179] HATs can also directly acetylate transcription factors, thereby altering their transcriptional activity.[180] At the present time it is not known if CBFA2 is acetylated by the bound HATs.

In addition to the RHD and transcriptional activation domains, CBFA2 contains several other functional motifs that are important for its biological activity (Figure 1.5). These include:

(1) A proline-serine-threonine-rich (PST) region which has been shown to be essential for transcriptional activation [181; 182]. This region contains potential phosphorylation sites for the extracellular signal-regulated kinase pathway.[183]

(2) A 31-amino-acid nuclear matrix attachment site (NM).[184; 185]

(3) A second weak transcriptional activation domain encompassing the PST and NM domains.

(4) Two putative transcriptional repression domains:

(4a) The first of the repression domains corresponds to an 80-amino-acid domain immediately C-terminal to the RHD that has been found to bind Ear2 (an orphan member of the nuclear hormone receptor superfamily).[186; 187]

(4b) The second repressor domain corresponds to a region in the C-terminal portion of the protein (Figure 1.5).[188]

(5) The C-terminal 5 amino acids, VWRPY, are conserved among all CBFα family members and have been shown to function as a binding site for the transcriptional co-repressor Groucho (Figures 1.4 and 1.5).[186; 189] By binding Groucho or the related mammalian homologues TLE1-4, CBFA2 changes from a transcriptional activator to a repressor.[190]

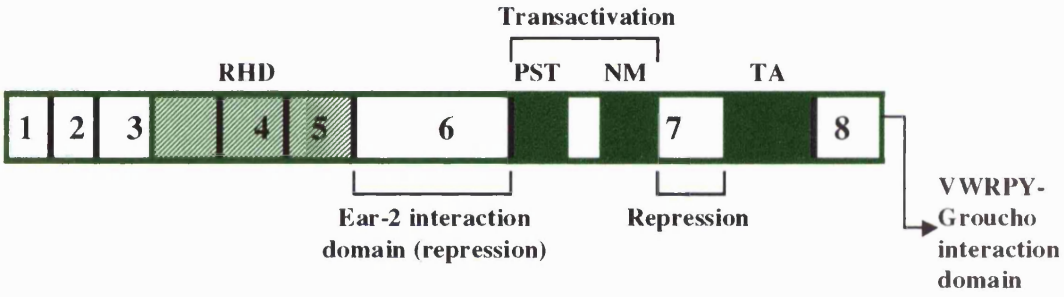


Figure 1.5. Structural Organisation of the CBFA2 protein.

The numbers represent the 8 exons. Functional regions shown are the Runt homology domain (RHD), a proline-serine-threonine-rich region (PST), a nuclear matrix attachment sequence (NM), and a C-terminal transcriptional activation (TA) domain. A second transcription activation domain has also been localised to a region that encompasses both the PST and NM motifs. In addition, the region of CBFA2 C-terminal to the RHD also contains two repression domains, with the more N-terminal domain interacting with Ear-2. The C-terminal end of CBFA2 ends in the amino acid sequence VWRPW (single amino acid code), which binds the Groucho co-repressor. Adapted from ref. [174]

Alternatively spliced forms of CBFA2 have been identified, some of which lack exons 7 and 8 containing the transcriptional activation domain.[181; 182; 191-193] Although these alternatively spliced forms comprise only a minority of CBFA2 transcripts; changes in the ratio of different isotypes may lead to profound changes in the transcriptional activity of the CBFA2/CBFβ complex. CBFA2 isoforms that lack the transcriptional activation domain have been shown to have a higher DNA-binding affinity, but to be unable to activate transcription.[181; 182; 191] Expression of these isoforms would therefore result in transcriptional repression of CBFA2 targets. Consistent with this prediction is the observation that G-CSF-induced differentiation of the myeloid cell line 32Dcl3 can be blocked by CBFA2 isoforms that lack either transcriptional activation sequences C-terminal to the RHD [182] or N-terminal sequences that lack part of the RHD.[193]

These data suggest that expression CBFA2/CBFβ could lead either to transcriptional activation or repression, depending on the specific genes being regulated, the isoform of CBFA2 expressed, and the cellular context in which this occurs. If CBFA2 binds to transcriptional co-activators, then transcriptional activation will result. Alternatively, if CBFA2 is expressed as an isoform that cannot bind co-activators, or in cells that express high levels of co-repressors such as Groucho or Ear-2, then CBFA2 would function as a transcriptional repressor. In addition, the interaction of CBFA2 with both transcriptional co-activators and co-repressors may be further regulated by post-translational modifications of each component. Alterations in the balance of positive and negative signals that are mediated through the CBFA2/CBFβ complex are likely to contribute directly to haematopoietic cell development and transformation. This has been confirmed by using gene-targeting experiments. The *Cbfa2* gene has been inactivated in the germline of mice by homologous recombination and shown to be essential for definitive haematopoiesis of all lineages. Homozygous null animals, i.e. animals with no functional Cbfa2 protein, display normal morphogenesis and yolk sac-derived erythropoiesis, but die between embryonic days 11.5 and 12.5 due to CNS haemorrhage. The defect was shown to be intrinsic to the haematopoietic system by demonstrating that Cbfa2-null embryonic stem (ES) cells were unable to contribute to any haematopoietic lineage in chimeric mice.[194; 195] Furthermore, it has recently been demonstrated that this haematopoietic defect can be rescued by expressing *Cbfa2* under the control of

endogenous *Cbfa2* regulatory elements through targeted insertion. The rescued *Cbfa2*$^{-/-}$ ES cell clones contributed to lympho-haematopoiesis within the context of chimeric animals. Rescue was shown to require the transactivation domain of Cbfa2 but not the C-terminal VWRPY Groucho binding motif (Figure 1.5).[196] Given these results, it is not surprising that the *CBFA2* gene is the most common target of chromosomal translocations in acute leukaemia.[197] These abnormalities will be discussed in detail at a later stage. Furthermore, it has recently been reported that haploinsufficiency of CBFA2 is responsible for an autosomal dominant congenital platelet defect and predisposes to the development of leukaemia.[198]

1.3.2. CBFA1

The *CBFA1* gene, identified as being homologous to the murine *Cbfa1* gene, was first cloned in 1994 and mapped to chromosome 6p21.[146] In addition to the highly conserved RHD, extensive sequence similarities to *CBFA2* were observed in other parts of the protein. These include an identical putative ATP-binding site, a C-terminal PST domain, and the VWRPY Groucho interaction domain.[146; 174]

Murine *Cbfa1* gene expression is initiated in the mesenchymal condensations of the developing skeleton and is strictly restricted to cells of the osteoblast lineage. Cbfa1 binds to, and regulates the expression of multiple genes in osteoblasts. The forced expression of Cbfa1 in non-osteoblastic cells induces the expression of osteoblast-specific genes.[199] Mice lacking both copies of the *Cbfa1* gene are completely deficient in bone formation, due to a defect in osteoblast differentiation.[200; 201]

The human homologue, *CBFA1*, has been implicated in a genetic disorder, cleidocranial dysplasia (CCD); an autosomal dominant trait characterised by moderate skeletal malformations.[202] The mutations in CCD patients inactivate only one copy of the *CBFA1* gene, indicating that the disorder is caused by haploinsufficiency of CBFA1.

1.3.3. CBFA3

The *CBFA3* gene has been mapped to chromosome 1p36. Similar to the *CBFA2* gene product, CBFA3 contains the RHD, the identical putative ATP-binding site, the C-terminal PST domain, and the VWRPY Groucho interaction domain.[146; 174]

CBFA3 is expressed predominantly in cells of haematopoietic origin.[203] Like CBFA2, CBFA3 has been shown to activate transcription of the TCRβ gene promoter. CBFA3 forms a complex with Smad3, a receptor-regulated signal transduction protein for members of the transforming growth factor-β (TGF-β) superfamily, and stimulates transcription of the germline Ig Cα promoter[204]. It has recently been shown that, similar to CBFA2, CBFA3 is also capable of interacting with TLE1 and acting as a transcription repressor for T-cell receptor enhancers.[189] Based on these studies it is hyopthesised that CBFA3 may play a role in haematopoietic cell differentiation.

1.3.4. CBFβ

The predicted amino acid sequence of the CBFβ subunit does not reveal any known structural motif.[108; 141; 144] The human *CBFβ* gene has been mapped to chromosome 16q22. Two *Drosophila* proteins, named brother (Bro) and big-brother (Bgb), structurally and functionally homologous to CBFβ, have been cloned.[205] CBFβ, Bgb, and Bro increase the DNA-binding affinity of Runt 20-fold, and are also able to increase the DNA-binding affinity of the mammalian CBFα proteins, although to a lesser extent.[205] Currently the only function ascribed to the product of the *CBFβ* gene is to increase the affinity of CBFα binding for DNA, however, in contrast to members of the CBFα family, CBFβ appears to be ubiquitously expressed.[141; 205] The cellular localisation of the CBFβ subunits also differs in that the CBFα family are nuclear proteins whereas CBFβ remains in the cytoplasm and is only recruited to the nucleus upon heterodimerisation with the CBFα subunit.[206-208]

Germline disruption of the mouse *Cbfβ* gene produces effects in homozygous null mice similar to those seen in *Cbfa2*$^{-/-}$, indicating that Cbfβ is required for the function of Cbfa2 *in vivo*.[209] Interestingly the CBFβ gene is also a target for the inv(16)(p13;q22) seen in 15% of AML (M4eo).

It has now been shown that the CBFA2/CBFβ complex is frequently the target of chromosomal translocations in human leukaemias. These translocations will now be discussed in detail and are summarised in Figure 1.6.

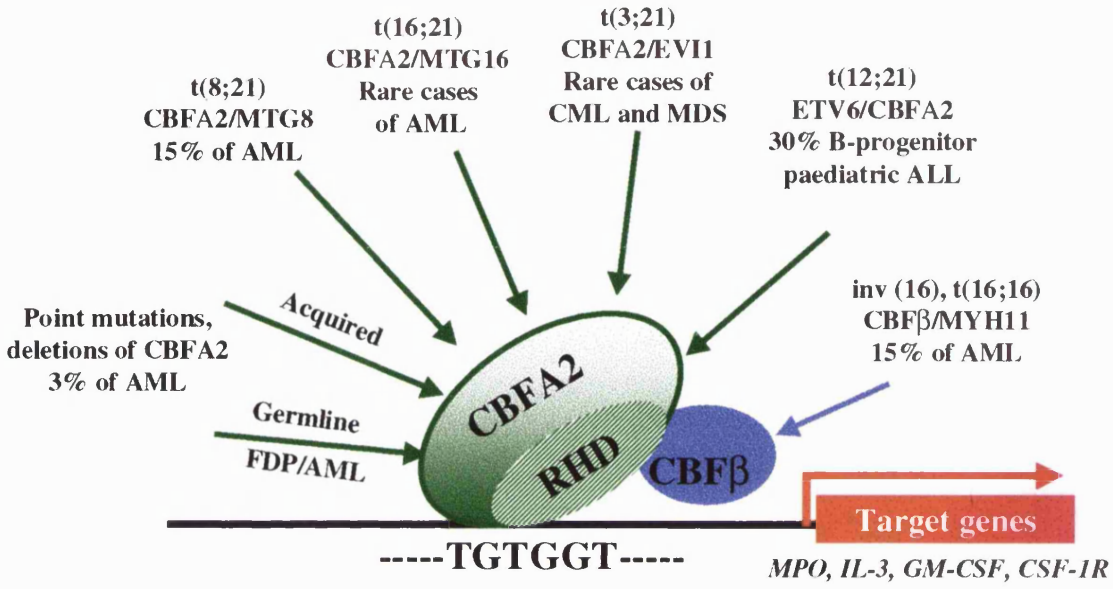


Figure 1.6. Molecular Consequences of Chromosomal Rearrangements that Target the CBFA2-CBFβ Transcription Factor Complex.

Adapted from ref.[2]

1.4. Chromosomal Translocations Involving the Core Binding Factor Complex

1.4.1. t(8;21)(q22;q22)

By far the most extensively investigated translocation is the t(8;21), through which the *CBFA2* gene was first identified, which occurs in ~40% of cases of AML,[104] most often FAB subtype M2. The *CBFA2* gene on chromosome 21 is transcribed from telomere to centromere, putting the t(8;21) fusion gene under the control of the *CBFA2* promoter on the derivative chromosome 8.

The *MTG8* (Myeloid Translocation Gene on chromosome 8, also called *ETO/CDR*) gene forms the 3' component of this fusion protein.[210; 211] In this translocation the first five exons of the *CBFA2* gene, containing the RHD are fused to almost the entire *MTG8* gene (Figure 1.7).

Initial sequence analysis demonstrated that *MTG8* is the mammalian homologue of the *Drosophila* gene *nervy*.[212] Recent studies have subsequently identified three other mammalian members of this family, *MTGR1*, *MTG16* and *ETO-2*.[105; 213] Amino acid sequence comparison between MTG family members and *nervy* reveals four evolutionarily conserved domains (Figure 1.7).

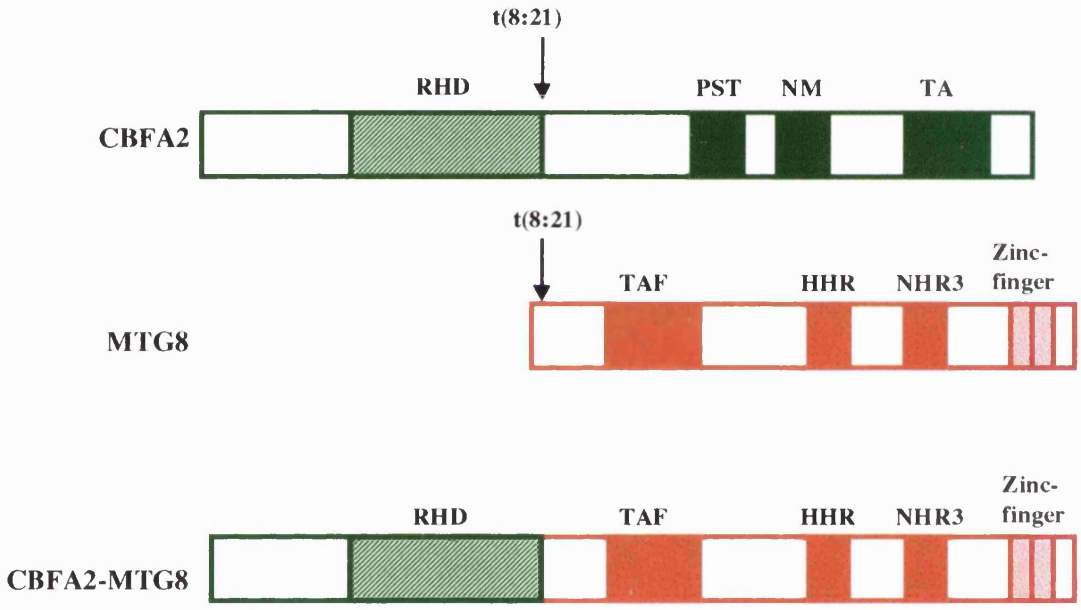


Figure 1.7. Schematic Representation of Full-Length CBFA2, MTG8 and CBFA2-MTG8 Fusion Proteins.

The runt homology (RHD), proline-threonine-serine rich (PST), nuclear matrix attachment (NM), and transactivational (TA) domains of CBFA2 are shown. The MTG8 protein contains four regions that have high homology to the Drosophila protein Nervy and to the MTG family members MTGR1, MTG16 and ETO-2. These regions include an N-terminal domain with homology to transcriptional-activating factors (TAF), a hydrophobic heptad repeat (HHR), a small region with homology to MTG proteins referred to as the Nervy homology region 3 (NHR3), and a C-terminal domain that contains two Zn-finger motifs. Adapted from ref. [174]

Importantly, MTG16 was identified as the target of the t(16;21)(q24;q22) translocation.[105] This is a much rarer but recurrent chromosomal abnormality associated with therapy-related myeloid malignancies.[214] This translocation has been shown to link the *MTG16* gene to *CBFA2*, producing a CBFA2-MTG16 fusion protein whose structure is similar to CBFA2-MTG8.[105] Identification of a second MTG family member involved in a translocation with CBFA2 suggests that MTG sequences are critical for the transforming activity of these fusion oncoproteins.

MTG8 is expressed as a nuclear phosphoprotein in brain and CD34+ haematopoietic progenitor cells, whereas MTGR1 and ETO-2 are ubiquitously expressed.[154; 213; 215] Although MTG8 is a nuclear zinc-finger-containing protein there is no experimental evidence to suggest that MTG8 can bind directly to DNA. Nevertheless, the structure of MTG8 would suggest that it is likely to function as a transcriptional regulator. This hypothesis is supported by recent experiments demonstrating that MTG8 can directly interact with the nuclear co-repressors N-CoR and Sin3A, and through these interactions can recruit an active histone deacetylase (HDAC).[216-218] The co-repressor proteins N-CoR and Sin3A bind to separate regions of MTG8[217] suggesting that MTG8 may function as an adapter protein within a nuclear co-repression complex. This function may stabilise the interaction of these co-repressors and tether them to sequence-specific DNA-binding transcription factors or, alternatively, recruit these factors away from other transcription proteins and thus inducing a fundamental change in transcriptional activity.

In addition to interacting with transcriptional co-repressors, MTG family members have also been shown to form homo- and heterodimers.[213; 215] Dimerisation is mediated through the hydrophobic heptad repeat (HHR) region and does not appear to interfere with the ability of these proteins to interact with N-CoR, Sin3A, or HDAC. Thus, the MTG8 family members are likely to form multi-subunit complexes that function in transcriptional regulation. The formation of different heterodimers may lead to significant functional differences in the activity of these complexes.

The CBFA2-MTG8 fusion protein retains many of the important functional domains of both CBFA2 and MTG8, including the RHD of CBFA2 and MTG8 sequences that have been shown to mediate homo- and heterodimerisation with MTG family members as well as interaction with nuclear co-repressors (Figure 1.7). As predicted from its structure, CBFA2-MTG8 continues to bind the core enhancer sequence and to heterodimerise with CBFβ.[143] Similarly, like wild-type CBFA2, the fusion protein

regulates the nuclear accumulation of CBFβ; but some of its critical functions differ. First, the transcriptional activation domains of CBFA2 are deleted and replaced by MTG8 sequences known to interact with nuclear co-repressors, indicating that the chimeric protein should function not as a transcriptional activator, but as a transcriptional repressor (Figure 1.8). This hypothesis has been confirmed by the finding that CBFA2-MTG8 directly represses CBFA2-mediated transcriptional activation in transient transcription assays.[171; 219; 220] Second, CBFA2-MTG8 binds CBFβ more avidly than CBFA2, and therefore accumulates CBFβ more efficiently in the nucleus than wild-type protein.[221]

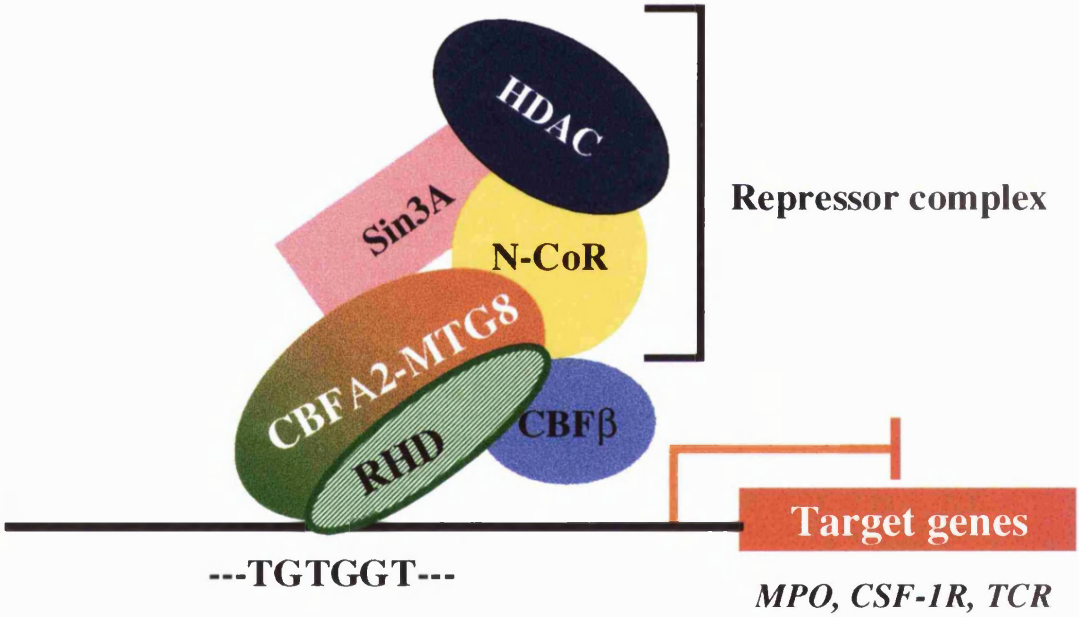


Figure 1.8. The CBFA2-MTG8 Fusion Protein Retains the Ability to Bind to the Core Enhancer Sequence and to Heterodimerise with CBFβ.

The fusion protein, unlike wild type CBFA2, binds through MTG8 sequences to other MTG family members such as MTG16, MTGR1 and ETO-2 as well as to co-repressor complex containing N-CoR, Sin3A and HDAC. This latter interaction results in the repression of genes whose transcription is normally activated by CBFA2/CBFβ.

The activity of CBFA2-MTG8 is likely to be modified by the ability of this chimeric protein to homo- and heterodimerise with MTG family members through the HHR domains of MTG8. Recent data demonstrate preferential dimerisation with the ubiquitously expressed MTG family member MTGR1, an interaction that augments CBFA2-MTG8-mediated repression of CBFA2-dependent transcription.[213]

Although the majority of data suggests that CBFA2-MTG8 functions as a transcriptional repressor, it has also been found to activate transcription of the *BCL-2* promoter through a CBFA2-binding site that resides within a negative regulatory region of the promoter.[222] Similarly, CBFA2-MTG8 can synergise with CBFA2 to activate the *CSF-1R* promoter.[223] The mechanism by which CBFA2-MTG8 activates transcription remains to be determined and is still controversial.[224] However, these data raise the possibility that the activity of CBFA2-MTG8 is both promoter and cell-type dependent.

Taken together these data suggest a model in which CBFA2-MTG8 induces haematopoietic cell transformation by: (1) actively repressing CBFA2-mediated transcriptional activation, thereby blocking the normal activity of CBFA2, (2) repressing transcription by other CBFA family members, (3) interfering with the normal function of MTG8 and other MTG family members, and (4) aberrantly activating the transcription of CBFA2- regulated and novel CBFA2-MTG8-specific target genes.

To directly investigate the role of CBFA2-MTG8 in leukaemogenesis, gene targeting has recently been used to create a *CBFA2-MTG8* "knock-in" allele that mimics the t(8;21). Unexpectedly, embryos heterozygous for the fusion gene (*CBFA2-MTG8*$^{+/-}$) died around E13.5 from a complete absence of normal foetal liver-derived definitive haematopoiesis.[225] This phenotype is similar to that seen following homozygous disruption of either *Cbfa2* or *Cbfβ* (described in 1.3.1 and 1.3.4).[194; 195; 209; 226; 227] However, in contrast to *Cbfa2*- or *Cbfβ*-deficient embryos, foetal livers from *CBFA2-MTG8*$^{+/-}$ embryos contained dysplastic multilineage haematopoietic progenitors with abnormally high self-renewal capacity *in vitro*. When the same group retrovirally transduced the *CBFA2-MTG8* fusion into murine adult bone marrow-derived haematopoietic progenitors *CBFA2-MTG8*-expressing cells were again found to have an increased self-renewal capacity and could be readily established into immortalised cell lines *in vitro*.[225] Taken together, these studies suggest that CBFA2-MTG8 not only neutralises the normal biologic activity of CBFA2 but also directly induces aberrant haematopoietic cell proliferation.

1.4.2. t(3;21)(q26;q22)

CBFA2 is also involved in a second, more rare recurring translocation, t(3;21)(q26;q22), which occurs mainly in patients with therapy-related AML or MDS who had been previously treated with drugs including topoisomerase II inhibitors, and in patients with CML-BC.[228] This translocation gives rise to the chimeric fusion genes *CBFA2-MDS1* *CBFA2-EAP* and *CBFA2-EVI1*.[197] In each case the breakpoint occurs between exons six and seven of the *CBFA2* gene (Figure 1.9). *EAP* (Epstein-Barr virus RNA-associated protein) codes for the ribosomal protein L22. However, the *EAP* reading frame is not maintained in the fusion with *CBFA2* and translation of *CBFA2-EAP* stops after the addition of 17 non-EAP-related amino acid residues to the RHD of CBFA2.[230] This shortened CBFA2 protein may dominantly interfere with normal CBFA2 function during myelopoiesis without a contribution from a partner protein.[231]

MDS1 (myelodysplasia syndrome) is a small gene that is centromeric to *EAP* and encodes a protein of 170 amino acids.[230] *CBFA2-MDS1* contains the same 5' *CBFA2* region as that found in *CBFA2-EAP*, fused in frame to *MDS1*. The function of MDS1 is unknown.

EVI1 encodes a DNA-binding protein with seven zinc-finger motifs at the N-terminus, three zinc-finger motifs in the distal third of the molecule, and an acidic domain at the C-terminus and is not normally expressed in bone marrow or haematopoietic cells.[232]

A variant fusion transcript that includes the *MDS1* sequence fused between the *CBFA2* and *EVI1* sequences has also been reported in leukaemic and normal cells.[230; 233]

The inclusion of both the runt DNA-binding/heterodimerisation domain of CBFA2 and the zinc-finger DNA-binding domains of EVI1 in the CBFA2-EVI1 and CBFA2-MDS1-EVI1 fusion proteins, afford these proteins striking structural similarities to the CBFA2-MTG8 fusion product (Figure 1.8). CBFA2-EVI1 and CBFA2-MDS1-EVI1 can interfere with CBFA2-mediated transactivation.[231; 234] 32D cl3 cells expressing CBFA2-MDS1-EVI1 undergo cell death without differentiation, mimicking the effect of EVI1 alone.[234] Also, CBFA2-MDS1-EVI1 and EVI1 prevent TGFβ-mediated growth inhibition of 32D cl3 cells and other cell types.[235; 236] This effect depends on the integrity of the first zinc-finger cluster and of a repression domain located between the zinc finger clusters.[235; 236] Interference with TGFβ-mediated growth inhibition can be accounted for by interaction of CBFA2-MDS1-EVI1 or EVI1 with Smad3, a downstream effector of

TGFβ signalling.[235-237] Furthermore, CBFA2-EVI1 accumulates CBFβ in the nucleus more efficiently than wild-type CBFA2.[221]

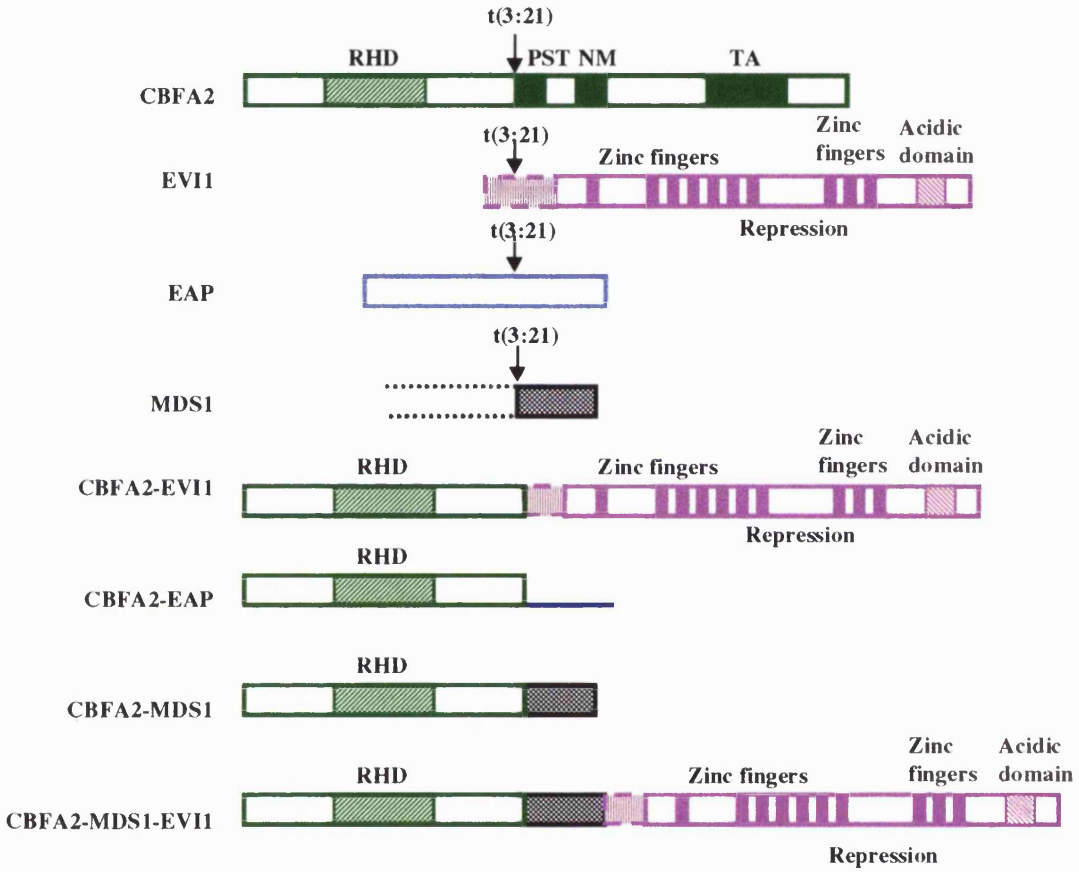


Figure 1.9. Diagrammatic Illustration of the Proteins Involved with CBFA2 in the Chimeric Fusion Products Generated by the t(3;21) Translocation.

The arrows mark the site of the fusion of CBFA2 with the partner protein after the translocation. The fusion of CBFA2 with EAP is not in frame, and a short blue line in the CBFA2-EAP fusion product indicates the 17 non-EAP-related amino acids. The unknown amino end of MDS1 is shown as a dashed box. The fusion of CBFA2 with EVI1 includes the second untranslated exon of EVI1, marked as a dashed line with vertical bars. Known structural motifs are indicated. The proteins are not drawn to scale. Adapted from ref.[197]

Recently, the effect of the *CBFA2-MDS1-EVI1* fusion gene *in vivo* has been analysed by retrovirally transducing the chimeric gene into mouse bone marrow cells. The mice suffered from AML 5-13 months after transplantation with retrovirally transduced bone marrow cells. The disease could be readily transferred into secondary recipients and resulted in a shorter latency of the leukaemia.[238]

Thus, not only are the fusion products generated by the t(3;12) translocation strikingly similar to the CBFA2-MTG8 product of the t(8;21) translocation but they may also contribute to leukaemogenesis in a similar way by inhibiting normal CBF function and by independent effects of the MDS1/EVI1 domain.

1.4.3. Inv(16)(p13;q22)

The importance of CBF in acute leukaemias is further demonstrated by chromosomal abnormalities involving the CBFβ subunit. Inv(16)(p13;q22) or the less common t(16;16)(p13;q22) are present in 10% of AML, usually FAB M4eo. The translocation leads to the CBFβ-MYH11 fusion product. The majority of CBFβ is fused to the tail domain of MYH11 (also known as smooth muscle myosin heavy chain, SMMHC) (Figure 1.10).[108] Several variants of CBFβ-MYH11 RNA and protein have been detected in cases of inv(16).[108; 239; 240] The most common variant includes 165 CBFβ residues and 446 MYH11 residues and is detected as a 70 kDa protein. The MYH11 domain is α-helical and consists of multiple, related 28 amino acid regions. One face of the α-helix is hydrophobic, allowing dimerisation. The other face is hydrophilic, with alternating positively and negatively charged zones. This face mediates multimerisation, which occurs with a 98 amino acid (3.3 repeat) stagger.[241] In addition MYH11 has a non-helical C-terminal tail. Human MYH11 has two isoforms, MYH11204 and MYH11200, which differ the length of their non-helical C-termini as a result of alternative splicing.[242] CBFβ-MYH11204 is more highly expressed than the CBFβ-MYH11200 isoform in M4eo AMLs.[240]

CBFβ-MYH11 can interfere with CBF DNA-binding by sequestering CBFα subunits in complexes formed as a result of multimerisation via the MYH11 domain.[240; 243] In leukaemic blasts, CBFβ-MYH11 was detected in small nuclear speckles, and at high concentration CBFβ-MYH11 forms rod-like structures in fibroblastic and haematopoietic cell lines.[240; 243; 244] The relevance of these structures in leukaemogenesis

is unknown. CBFβ-MYH11 has been shown to sequester CBFα subunits in the cytoplasm of adherent cell lines.[245; 246] This may result from increased affinity of CBFβ-MYH11 for the cytoskeleton, compared with CBFβ, possibly as a result of interaction of its MYH11 segment with cytoskeletal-associated non-muscle myosins.[247] CBFβ-MYH11:CBFα complexes retain the ability to bind DNA allowing the possibility that the fusion gene may also interfere with CBFα trans-activation via local effects on promoter/enhancer transcription complexes.[108; 243]

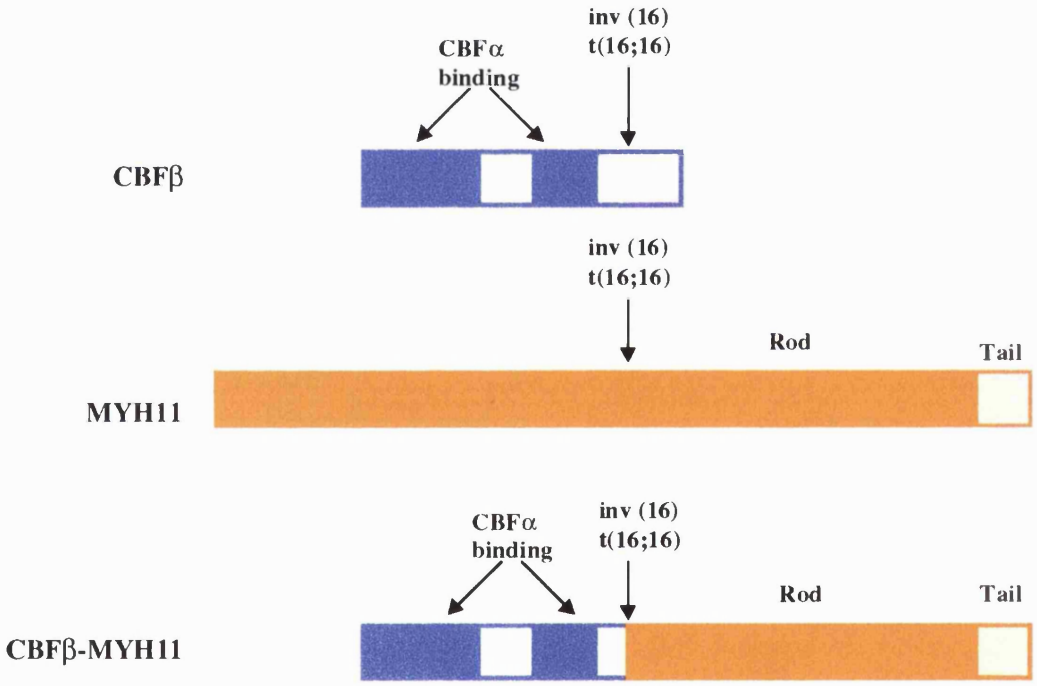


Figure 1.10. Schematic Representation of CBFβ, MYH11 and CBFβ-MYH11 Proteins.

The position of the most commonly identified breakpoint identified in inv(16) and t(16;16) is indicated. The CBFα binding domains of CBFβ and the α-helical and C-terminal tail domains of MYH11 are shown.

Via one or more of these mechanisms, CBFβ-MYH11 interferes with CBF function.[245; 246; 248] Deletion of 11 N-terminal CBFβ residues, required for CBFα interaction, as well as 283 C-terminal residues from the MYH11 segment, required for dimerisation, prevented CBFβ-MYH11 from interfering with CBFα DNA-binding and trans-activation.[248] Although the mechanism by which the CBFβ-MYH11 fusion oncoprotein contributes to cellular transformation has not been fully elucidated it may act in a similar manner to the previously described fusion oncoproteins containing CBFA2 by inhibiting normal CBF function and by independent effects of the MYH11 domain. Recently, gene targeting has been used to create a *CBFβ-MYH11* "knock-in" allele that mimics the inv(16). Mouse embryos heterozygous for *CBFβ-MYH11* lacked definitive haematopoiesis and developed multiple fatal haemorrhages around E12.5.[249] This phenotype is very similar to that resulting from homozygous deletions of *cbfa2* and *cbfβ*.[194; 195; 209; 226; 227] Chimeric mice were leukaemia free, but the "knocked-in" *CBFβ-MYH11* allele was only identified in erythrocytes, not leukocytes, in the circulating blood.[249; 250] These results indicate that haematopoietic stem cells containing the *CBFβ-MYH11* gene are present in the chimera's bone marrow, which have a selective defect in lymphoid and myeloid differentiation. *CBFβ-MYH11* chimeric mice did not develop tumours in their first year indicating that *CBFβ-MYH11* may contribute to leukaemic transformation but additional genetic events are likely to be required. To test this hypothesis, 4-16 week old *CBFβ-MYH11* chimeric mice were injected with a single sub-lethal dose of N-ethyl-N-nitrosourea (ENU), a potent DNA alkylating mutagen. 84% of the treated *CBFβ-MYH11* chimeric animals developed leukaemia 2-6 months after treatment, whereas none of the ENU-treated control mice developed leukaemia.[250] These data indicate that *CBFβ-MYH11* blocks myeloid differentiation and predisposes mice to leukaemia The tumours in the *CBFβ-MYH11* chimeras were almost exclusively AML M4, even though ENU mutagenises cells in many tissues and *cbfβ* is broadly expressed,[144] suggesting a strong disease specificity for the *CBFβ-MYH11* oncogene. Furthermore, alteration of other critical genes is necessary to trigger leukaemogenesis.

The CBF complex was generally considered to be a target for chromosomal translocations in myeloid cells until the *CBFA2* gene was found to be joined to *ETV6* in B-lineage ALL.

1.5. ETV6

The partner of t(12;21) on chromosome 12 (p13) was initially identified in 1994 as the translocated ETS leukaemia (*TEL*) gene involved in the t(5;12)(q33;p13).[92] This gene has latterly been renamed ETS-type variant 6 (*ETV6*). The ETS (E26-transformation specific) family of transcription factors is a large group of evolutionarily conserved transcriptional regulators that play an important role in a variety of cellular processes throughout development and differentiation (reviewed in [251]). All ETS proteins bind DNA via a highly conserved ~85 amino acid region, the ETS domain, that recognises a purine-rich GGAA/T core motif within promoters and enhancers of various genes (reviewed in [252]). In addition to sequence recognition, DNA binding may also be regulated through phosphorylation of ETS proteins and by protein-protein interactions mediated via other domains (eg, the "pointed" [PNT] domain) within ETS proteins.[253].

Although expressed in a variety of tissues, most currently known *ETS* genes are expressed predominantly in haematopoietic cells and many are key regulators of blood cell development and differentiation.[254]

The *ETV6* gene consists of eight exons and, like one-third of ETS family transcription factors, contains the N-terminal PNT domain (which mediates dimerisation) and a C-terminal DNA-binding ETS domain (Figure 1.11).[92] The PNT domain of ETV6 is capable of mediating homodimerisation.[255; 256] This domain is also necessary for interaction with another ETS factor, Fli-1, and interferes with the trans-activation of the GPIX promoter by Fli-1.[257] Furthermore, the PNT domain has recently be shown to be required for the interaction with the ubiquitin-conjugating enzyme UBC9.[258] By analogy to other members of the ETS family, it is likely that ETV6 is also a DNA-binding transcription factor. The nature of the genes regulated by ETV6 is not known, but recent studies have shown that ETV6 may act as a transcription repressor.[258; 259] ETV6 contains two domains that can independently repress transcription of a reporter gene. The two domains are the PNT, located at the N-terminus, and a central region of the protein located between the PNT and the ETS domains (Figure 1.11).[259]The central region represses transcription by interaction with the co-repressors mSin3A, SMRT and N-CoR. The central region, but not the PNT domain, is sensitive to inhibitors of histone deacetylases. In contrast to the central region of ETV6, the PNT domain represses transcription by a mechanism that does not involve co-repressors sensitive to inhibitors of

histone deacetylases. Because the PNT domain is a protein interaction domain, it is likely that other, as yet unidentified, proteins involved in repression, may be recruited.

Unlike the majority of ETS proteins, ETV6 is ubiquitously expressed. The mouse homologue is also widely expressed and mice in whom both *Etv6* alleles have been deleted die between E10.5-11.5. These mice have normal yolk sac haematopoiesis, but foetal and adult haematopoiesis and lymphopoiesis could not be assessed. However, analysis of chimeric mice showed that *Etv6*$^{-/-}$ cells did not contribute to bone marrow haematopoiesis, although these cells contributed normally to yolk sac and foetal liver myeloid and erythroid progenitors. [260] These studies suggest that the ETV6 protein may be required for haematopoietic cells to home to the bone marrow but not for their differentiation.

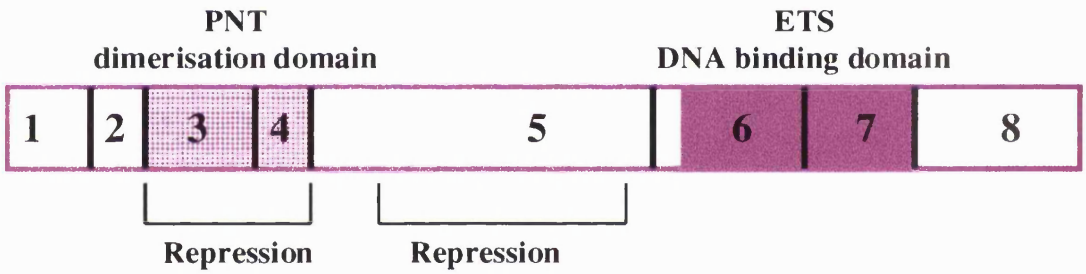


Figure 1.11. Structural Organisation of the ETV6 Protein.

The eight exons are numbered. Functional regions shown are the pointed (PNT) dimerisation and the ETS DNA-binding domains. Also shown are the two repression domains.

Recently, a novel *ETS* gene has been characterised that is highly homologous to *ETV6* called *TEL2*. This gene was discovered via its homology across the ETS domain and it has been localised on chromosome 6p21. Unlike the ubiquitously expressed *ETV6* gene, however, *TEL2* expression appears to be restricted to the haematopoietic tissues. The TEL2 protein associates with itself and with ETV6 in doubly transfected Hela cells and this interaction is mediated through the PNT domain of ETV6.[261]

1.6. Chromosomal Translocations Involving ETV6

Translocations involving the *ETV6* gene are associated with many different human leukaemias in which ETV6 is fused to several different proteins.

1.6.1. t(12;21)(p13;q22)

As previously mentioned the cytogenetically cryptic (12;21) translocation encoding the ETV6-CBFA2 fusion protein is detected in approximately 25% of case of childhood ALL, making this the most common genetic abnormality in lymphoid leukaemias.[2] The t(12;21) results in fusion of the first five exons (including the PNT domain of *ETV6*) to the terminal seven exons of *CBFA2*, including the RHD, PST, NM, and TA domains (Figure 1.12).[1; 102]

ETV6-CBFA2 interferes with CBFA2 DNA-binding and represses activation of the TCRβ and IL-3 promoters by wild-type CBFA2. This repression is dependent upon the integrity of the PNT domain of ETV6, the RHD of CBFA2 and the amino acids 216-290 of CBFA2.[262; 263] The observation that the PNT domain of ETV6 can mediate heterodimerisation between ETV6-CBFA2 and ETV6 suggests that the chimeric molecule may also directly alter the normal function of the wild-type ETV6 protein.[89; 255; 256; 264]

Interestingly, the non-translocated *ETV6* allele is frequently deleted in cases of ALL with t(12;21).[265-267] Loss of heterozygosity at the *ETV6* locus is common in childhood ALL,[268-270] and the four ALL cases from which the *ETV6-CBFA2* fusion was first cloned all had deletions of the non-translocated *ETV6* allele.[1; 102] These results indicate that deletion of *ETV6* may be a secondary event in leukaemias with t(12;21) and suggests a consistent association between *ETV6-CBFA2* fusion and deletion of the normal ETV6 allele.[265] This raises the possibility that the *ETV6* gene could have tumour suppressor activity, although, mitigating argument against this is the failure to detect bi-allelic loss of *ETV6* in the absence of the t(12;21).[1; 265; 271; 272] In addition, not all cases with loss of *ETV6* contain the *ETV6-CBFA2* fusion.[265; 266] One hypothesis, for the role of *ETV6* deletions, is that the product of the normal *ETV6* allele interferes with the activities of ETV6-CBFA2 by interaction via the shared PNT dimerisation domain. Consistent with this model, several cases of ALL have been identified carrying small deletions within the PNT domain of the *ETV6* locus.[273] An alternative hypothesis is that loss of ETV6 itself

provides cells with a proliferative advantage. The defect in marrow homing identified in $Etv6^{-/-}$ mice might also provide ALL blasts containing a similar $ETV6^{-/-}$ phenotype with a proliferative advantage.

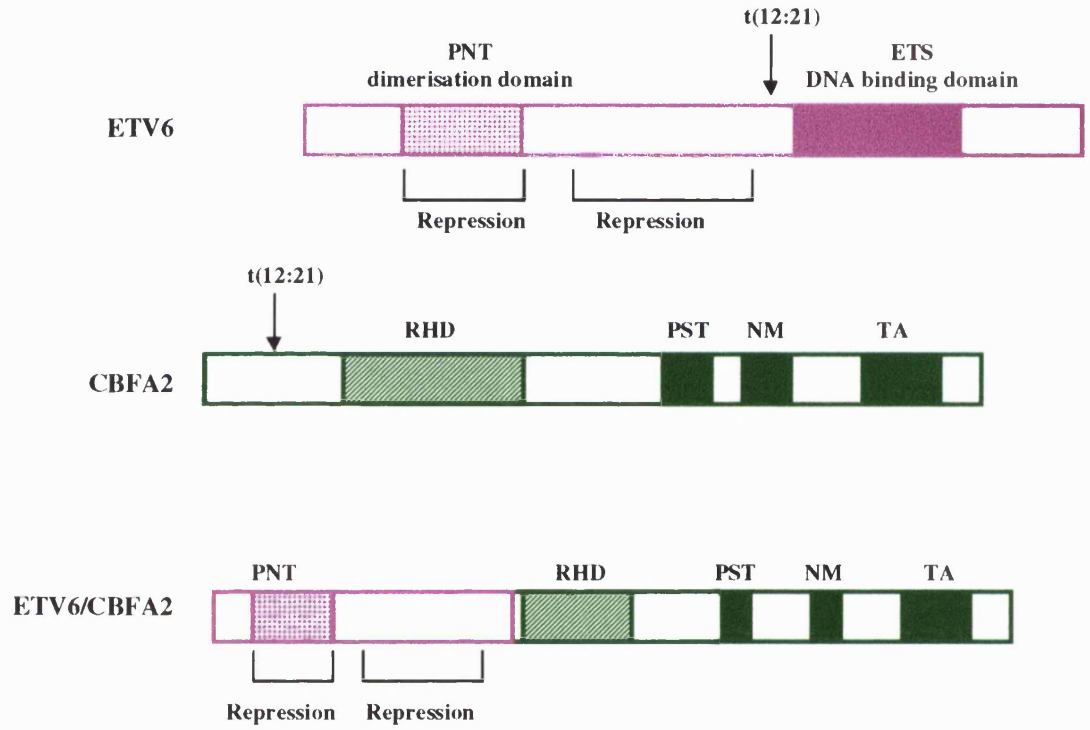


Figure1.12. Schematic Representation of Full Length ETV6, CBFA2 and ETV6-CBFA2 Fusion Proteins.

The t(12;21) breakpoint is indicated. The dimerisation (PNT) DNA-binding (ETS) and repression domains of ETV6 and the runt (RHD), a proline-serine-threonine-rich (PST), nuclear matrix attachment sequence (NM), and a C-terminal transcriptional activation (TA) domains of CBFA2 are shown.

Inactivation of normal ETV6 function, both through deletion of the non-translocated allele and disruption of function via fusion to CBFA2 is likely to contribute to the pathogenesis of the ETV6-CBFA2-associated leukaemias.

The acquisition of secondary events contributing to the progression *of ETV6-CBFA2* associated ALL is supported by the finding of identical *ETV6-CBFA2* fusion sequences in the lymphoblasts of two sets of identical twins. The first twin of each pair developed ALL at a much earlier age than the second twin (a 1.5 and 9 year interval).[274; 275] However, analysis of DNA from an archival slide from the twin diagnosed at 14 years identified a clone which contained an identical *ETV6-CBFA2* fusion product when analysed molecularly to the twin diagnosed at 5 years. Moreover, this clone was present 9 years before clinical diagnosis. These data suggest that the *ETV6-CBFA2* fusion could be generated in utero. The long latency period between the generation of the fusion and the development of leukaemia suggests that secondary events are required for the development of the leukaemic phenotype.

1.6.2. t(5;12)(q33;p13)

ETV6 was first identified as part of the *ETV6-PDGFRβ* (platelet derived growth factor receptor β) fusion that is formed by the t(5;12)(q33;p13) in chronic myelomonocytic leukaemia (CMML).[92] Normally, binding of PDGF to wild type PDGFRβ leads to dimerisation of PDGFRβ, activation of its tyrosine kinase, and autophosphorylation. Subsequent signal transduction pathways affect the mitogenic properties of the receptor. As a result of the t(5;12), however, the first four exons of ETV6, including the PNT domain, are fused, in-frame, to the PDGFRβ transmembrane and tyrosine kinase domains (Figure 1.13). The PNT domain of ETV6 then causes dimerisation and constitutive activation of the PDGFRβ kinase.[256; 264] Cooperation of the PNT domain of ETV6 and the tyrosine kinase activity of PDGFRβ has been shown by using Ba/F3 cells in which transformation depends on the fusion protein to be present.[256; 264]

Recently, the *ETV6-PDGFRβ* fusion gene has been expressed in haematopoietic cells of transgenic mice under the control of the human CD11a or the lymphoid-specific immunoglobulin enhancer promoter. Transgenic founders and their offspring expressed the transgene specifically in haematopoietic tissues and developed a myeloproliferative

syndrome or lymphomas of both T and B lineage.[276; 277] Treatment of ETV6-PDGFRβ transgenic animals with a protein kinase inhibitor with *in vitro* activity against PDGFRβ resulted in suppression of disease and prolongation of survival.[277] This murine model implicates the ETV6-PDGFRβ fusion protein in pathogenesis.

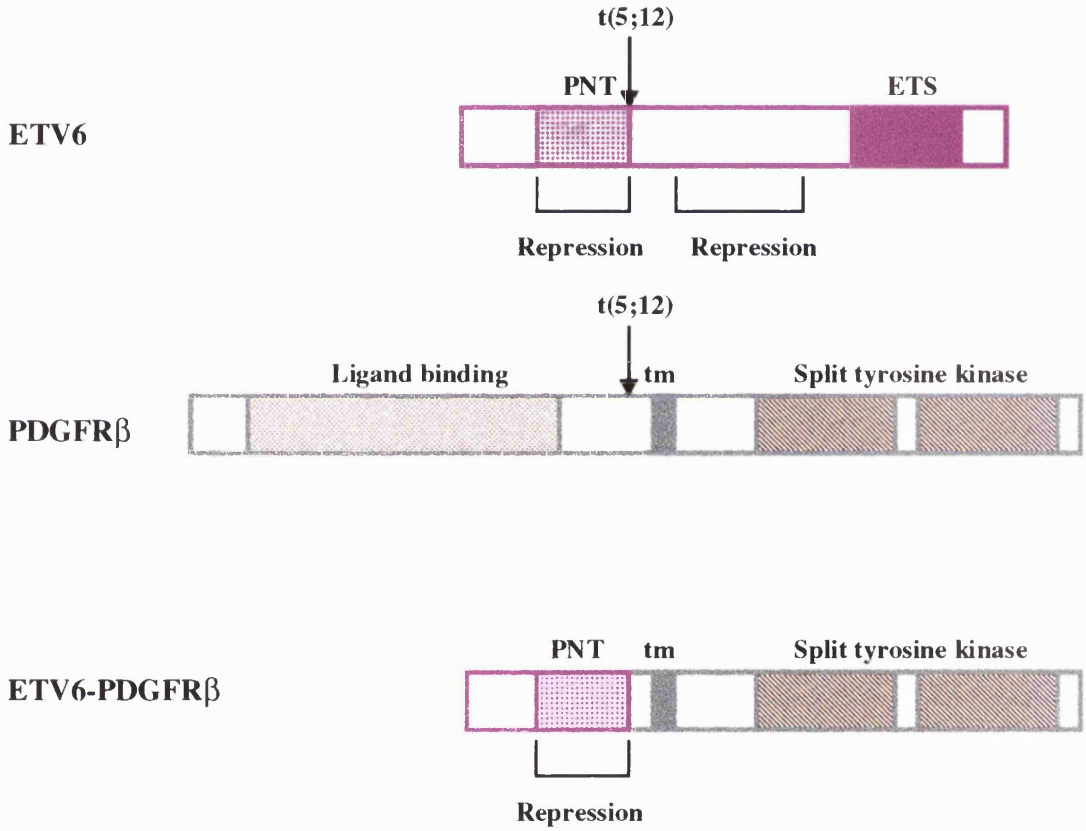


Figure 1.13 Schematic Representation of ETV6, PDGFRβ and ETV6-PDGFRβ Fusion Proteins.

The t(5;12) breakpoint is indicated. The PNT, ETS and repression domains of ETV6 and the ligand binding and split tyrosine kinase domains of PDGFRβ are shown. Tm indicates the transmembrane domain of PDGFRβ.

1.6.3. t(9;12)(q34;p13)

The fusion of ETV6 to a tyrosine kinase also occurs as a result of the t(9;12)(q34p13), which has been observed in cases of AML, atypical CML, and ALL.[87-89] This translocation fuses the region encoding the PNT domain of *ETV6* to the *ABL* proto-oncogene, which is also involved in the BCR-ABL fusion of ALL and CML (1.2.1.2.1.).[87-89] As in the case of ETV6-PDGFRβ, the PNT domain of ETV6 most likely serves as a dimerisation motif, leading to constitutive ABL tyrosine kinase activity.[89] Consistent with this hypothesis, treatment of cell lines expressing the ETV6-ABL protein with the specific ABL tyrosine kinase inhibitor STI571 inhibited cell growth.[278] ETV6-ABL is constitutively phosphorylated when expressed in cell lines, confers IL-3-independent growth to Ba/F3 cells, and transforms haematopoietic cells in culture.[86; 89]

1.6.4. t(9;12)(p24;p13)

A third example of ETV6 fused to a tyrosine kinase has been recently cloned from two ALL cases containing the t(9;12)(p24;p13) and one CML case with the t(9;15;12)(q24;q15;p13).[94; 279] In all three cases, chimeric messages encoding ETV6-JAK2 fusion proteins were expressed. Like ETV6-PDGFRβ and ETV6-ABL, ETV6-JAK2 contained the PNT domain of ETV6 fused in-frame to the tyrosine kinase domain of JAK2. As with the other ETV6-tyrosine kinase fusion proteins, both the ETV6 PNT domain and the JAK2 tyrosine kinase domain of ETV6-JAK2 are required to transform Ba/F3 cells to IL-3 independence. Furthermore, following transplantation in syngeneic animals of mouse bone marrow cells infected with a MSCV-ETV6-JAK2 retrovirus, mice developed a mixed myeloid-lymphoid leukaemia with a short latency.[280] A second group have recently generated transgenic mice in which the ETV6-JAK2 complementary DNA was placed under the control of the EμSRα enhancer promoter. ETV6-JAK2 founder mice and their transgenic progeny developed fatal T-cell leukaemia at 4 to 22 weeks of age.[281] Because the same ETV6-JAK2 fusion gene was used in both studies, the basis for the difference in disease type is likely to originate either from the type of promoter used to drive ETV6-JAK2 expression (MSCV LTR vs EμSRα) or from the fact that retroviral-mediated infection in tissue culture is relatively unrestricted in terms of the cell lineage and differentiation stage of targeted cells. Both studies, however, demonstrate that ETV6-JAK2 is a powerful oncogene in vivo and that, within the lymphoid lineage, ETV6-JAK2

preferentially transforms T-cells. The ETV6-JAK2 fusion has been shown to result in the constitutive tyrosine phosphorylation of STAT1 and STAT5 (signal transducer and activator of transcription) in Ba/F3 cells[280; 282] and in leukaemic cells.[281] Constitutive activation of STAT proteins in ETV6-JAK leukaemic cells could therefore bypass normal signalling pathways controlled by specific receptors to induce their uncontrolled proliferation.

1.6.5. t(1;12)(q25;p13)

A further example of ETV6 being fused to a tyrosine kinase has recently been described in a patient with AML M4eo, positive for the *CBFβ-MYH11* rearrangement and carrying a t(1;12)(q25;p13). A novel fusion transcript was identified between the *ETV6* and the Abelson-related gene (*ARG*) on 1q25, resulting in a chimeric protein containing the PNT domain of ETV6 and the protein tyrosine kinase domain of ARG. The *ARG* gene encodes a non-receptor tyrosine kinase characterised by high homology with *c-ABL.* [90]

1.6.6. t(12;15)(p13:q25)

However, ETV6 involvement in human translocations appears not to be restricted to haematologic malignancies. Recently, a ETV6-neurotrophin-3 receptor tyrosine kinase (NTRK3) fusion was identified in congenital fibrosarcoma cases carrying the t(12;15)(p13;q25).[283] ETV6-NTRK3 contains the PNT domain of ETV6 fused to the protein kinase domain of NTRK3, suggesting, once again, that PNT-mediated dimerisation and constitutive kinase activation play a role in transformation by this fusion, similar to that described for ETV6-PDGFRβ, ETV6-ABL, and ETV6-JAK2. Furthermore, the t(12;15)(p13;q25) resulting in an *ETV6-NTRK3* chimeric transcript has recently been identified in an adult AML patient.[95] Retroviral transduction of the fusion variant seen in AML causes a rapidly fatal myeloproliferative disease in a murine bone marrow transplant model, whereas transduction of the variant seen in congenital fibrosarcoma causes a long latency, pre-B-cell lymphoblastic lymphoma.[284]

1.6.7. t(12;22)(p13;q11)

In contrast to the ETV6-tyrosine kinase fusions in which the PNT domain of ETV6 appears to be essential for transformation, the t(12;22)(p13;q11) fuses the *MN1*

gene in-frame to the ETS DNA-binding domain of *ETV6*.[96] MN1, a nuclear protein of unknown function, has features that suggest that it is involved in transcriptional regulation. The MN1-ETV6 fusion contains almost all of MN1 fused to the ETS domain of ETV6, and is under the control of the MN1 promoter.

1.6.8. t(3;12)(q26;p13)

The reciprocal translocation t(3;12)(q26;p13) described in seven patients with acute transformation of MDS or myeloproliferative disorders results in the intergenic splicing of the first two exons of *ETV6*, *MDS1* sequences, and exon two of *EVI1*.[97; 98] ETV6 thus contributes no known functional domain to the predicted chimeric protein and the oncogenic potential of the translocation could be the result of the *ETV6* promoter driving the transcription of *MDS1* and *EVI1*. Interestingly, similar fusion products containing *MDS1* and *EVI1* are formed in the t(3;21)(q26;q22) involving the *CBFA2* gene (1.4.2).[106; 230]

1.6.9. Other Translocations Involving ETV6

ETV6 is also involved in a variety of other translocations in ALL, AML, CML, and MDS. Whilst in some cases the partner gene has been identified, such as *CDX* in t(12;13)(p13;q12),[100] *BTL* in t(4;12)(q11-12;p13),[99] *ACS2* in t(5;12)(q31;p13),[101] and ARNT in t(1;12)(q21;p13),[91] many others have yet to be isolated (reviewed in ref. [285]).

In summary, it can be seen that genes encoding the CBF complex and the ETV6 protein are involved in a large number of reciprocal translocations and that in many of these cases the product of the fusion gene has been directly implicated in leukaemogenesis. Furthermore they have close homologues in genes controlling embryogenesis in *Drosophila* and other invertebrates, indicating their conservation in nature and importance to programs of early cell development.[286; 287] Although a diverse number of transcription control proto-oncogenes have been implicated in human leukaemias their essential functions can be linked to a fundamental step in cell growth, development, or survival.[6; 7] This has been confirmed for the CBF and ETV6 proteins by germline disruption of the murine gene homologues *Cbfa2*, *Cbfβ* and *Etv6*. In each case

the null-phenotype was embryonic lethal indicating the importance of each gene in early mouse cell development.[194; 195; 209; 260]

At the present time more than 10 transcriptional control genes have been shown to play critical roles in normal haematopoiesis and whilst some of these factors are lineage specific, others operate early in haematopoietic development. A further group is widely expressed but perform specific functions in restricted blood cell types, ostensibly by interacting with lineage-restricted proteins (Figure 1.14).[5; 288] As the CBF genes are included in this group and that the aim of this work is to generate a mouse model containing the *ETV6/CBFA2* fusion gene, it is worthwhile to briefly review the early stages of haematopoiesis during which these genes are playing a major role.

1.7. Haematopoietic Development.

Pluripotent haematopoietic stem cells (HSCs) are the source of all blood cells in the adult. However, paradoxically, they are not the first blood cells to develop in the embryo. The first blood cells are mesodermal in origin and appear in the yolk sac of mouse embryos at ~8 days post conception. These consist primarily of nucleated erythrocytes with a small number of granulocytes and macrophages.[289] This first, transient wave of "primitive" yolk sac haematopoiesis is shortly followed by the appearance of "definitive" haematopoietic progenitor cells. These cells are capable of differentiating into all adult blood cell lineages. Definitive haematopoietic cells emerge from several sites in the embryo: from the embryonic yolk sac; the omphalomesenteric and umbilical arteries; the embryonic splanchnopleure; and from the aorta, gonad, mesonephros (AGM) region.[290-295] The first long-term repopulating HSCs, which are capable of reconstituting an intact haematopoietic system when transplanted into lethally irradiated mice, appear at dpc 10.0 in the AGM region.[294; 295] Definitive haematopoietic progenitor cells and long-term repopulating HSCs then migrate to the foetal liver, where they further expand and differentiate into definitive nucleated erythrocytes and cells of the myeloid lineage.

Mutations or lack of several genes encoding transcription factors, such as Tal, Lmo2, and Gata-2, some of which were identified by their involvement in human translocations, have profound effects on the development of both primitive and definitive blood cell lineages (reviewed in [296]). Other genes (Pu.1, Myb, Eklf, Ikaros, Pax5, Gata-1,

E2A, and NF-E2) seem to be required for the development of a subset of definitive haematopoietic lineages but not the development of primitive blood cells in the yolk sac (Figure 1.14).

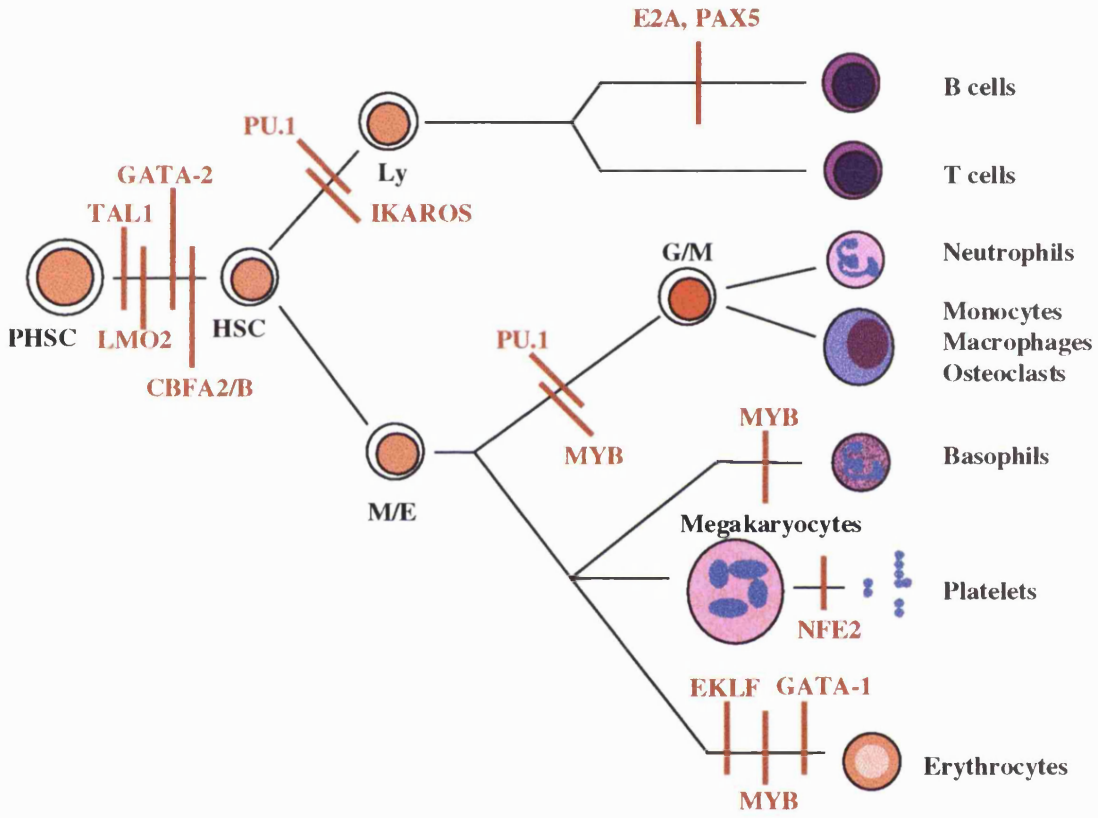


Figure 1.14. Schematic Diagram Illustrating the Stage at which Transcription Factors Influence Haematopoietic Development.

Factors serving as targets for chromosomal translocations in leukaemia and lymphoma are indicated in bold type. Note that transcription factor targets may be lineage specific (E2A) or uncommitted (CBFA2). HSC, haematopoietic stem cell; M/E, myeloid/erythroid progenitor; Ly, lymphoid progenitor; G/M, granulocyte,/macrophage progenitor. Adapted from ref. [288]

1.8. Experimental Studies to Investigate the Role Fusion Proteins Play in Tumourogenesis

As described in the previous sections, fusion proteins resulting from chromosomal translocations are frequently seen in leukaemias. In assessing the relevance of such proteins to the development of leukaemia, various experimental animal models have been used.

1.8.1. Transgenesis

Transgenesis is a method by which an animal gains new genetic information by the injection of foreign DNA. This method has been used to study the role of many fusion genes in leukaemogenesis (1.4, 1.6).[86; 225; 238; 280] The gene of interest is introduced randomly into the host's genome and therefore requires the presence of a promoter to guarantee its expression. Choosing a promoter that allows expression of the transgene in the appropriate cell type is difficult and even when the correct cell type is targeted, the chosen promoter will still not control expression in the same way as the endogenous promoter.

Acute promyelocytic leukaemia (APL) is associated with a reciprocal chromosomal translocation involving the retinoic acid receptor α (*RARα*) locus located on chromosome 17q21 fused to the promyelocytic leukaemia (*PML*) gene located on chromosome 15q21. A transgenic mouse has been generated in which the PML/RARα fusion protein is specifically expressed in the myeloid-promyelocytic lineage.[297] This was achieved by including a human cathepsin-G minigene (hCG) in the *PML/RARα* containing construct. The cathepsin G gene expression, whose products accumulate in the granules, found in the cytoplasm of myeloid progenitors and granulocytes, peaks at the promyelocyte stage of myeloid differentiation. Approximately 10% of the hCG-PML-RARa transgenic mice developed acute leukaemia with a differentiation block at the promyelocytic stage that closely mimics human APL. These findings illustrate that transgenesis can be a valuable tool in the study of chromosomal abnormalities providing suitable control elements are available.

1.8.2. Generating a Null ("Knock-Out") Phenotype by Homologous Recombination

Homologous recombination is a technique that overcomes the problems of transgenesis by introducing genes into the mouse genome to disrupt endogenous genes.[298] The function of a wild-type gene is disrupted by: a) insertion, or b) replacement. In the insertion model a positive-selection gene [which provides a growth advantage in selective media, such as neomycin (neo) or hygromycin (hyg)] is introduced within a functionally relevant exon of a gene. In the replacement model removing a relevant exon and replacing it with the same *neo* or *hyg* gene modifies the genomic organisation of the gene. A negative selection marker is introduced into the construct, usually the *thymidine kinase* (*TK*) gene of the herpes virus, to allow selection in the presence of gancyclovir. The whole construct is then injected and homologous recombination requires the replacement of the target sequence with the construct as a result of which the *TK* gene is lost. The gain of neomycin and gancyclovir resistance can enhance the selection for cells in which homologous recombination of the target sequence has occurred, with a frequency varying from target to target. The presence of the *neo* gene within an exon disrupts transcription, and thereby creates a null allele.[298] Embryonic stem (ES) cells are cells derived from the mouse blastocyst (an early stage of development, which precedes implantation of the egg in the uterus). If targeted homologous recombination occurs in an ES cell and this cell is injected into a recipient blastocyst, the resulting chimeric mouse will have some tissues derived from cells containing the manipulated gene. To determine whether the ES cells contributed to the germline, the chimeric mouse is crossed with a mouse that lacks the donor trait (usually coat colour). Any progeny that have the donor trait must be derived from the original, manipulated ES cell. In this way mice can be generated in which every cell contains the mutation. This technique has been successfully applied to the study of the role of the *Cbfa2*, *Cbfβ*, and *Etv6* genes in haematopoietic development (1.3.1,1.3.4,1.6.1).[194; 195; 209; 226; 227; 260]

1.8.3. Introducing New Genetic Material ("Knock-In") by Homologous Recombination

In addition to gene targeting to produce null mutations, homologous recombination can be used to mimic the consequence of chromosomal translocations. In this technique a construct is generated which, when introduced into the endogenous locus of interest by homologous recombination, creates an in frame fusion gene that utilises the

endogenous promoter to control expression. Unlike the "knock-out" model, the *neo* or *hyg* gene only provides a growth selective marker, rather than a method of gene inactivation, as described above. Furthermore, transcription and translation control sequences can be engineered into the construct, such as a poly-A addition sequence to allow for correct RNA processing. Negative selection is achieved by using the gancyclovir sensitisation by the *TK* gene as in the "knock-out" model.

A particular type of "knock-in" model has been applied to investigate the temporal contol of expression of a variety of genes. For example, a 5.4-kilobase cassette of non-homologous DNA has been introduced into the *int-2* locus in mouse ES cells. The inserted DNA contained a lacZ gene positioned to create an in-frame fusion with the int-2 protein-coding region. Upon differentiation of these cells to embryoid bodies, the int-2-lacZ fusion faithfully reproduced the expression pattern of int-2 RNA indicating that the artificial gene was under the control of the endogenous promoter.[299] Homologous recombination in ES cells has been used to create an in-frame fusion of the human *AF9* gene with exon 8 of the mouse *Mll* gene thus mimicking the translocation t(9;11)(p22;q23) associated with AML. The chimeric mice developed AML, the blast cells of which contained the *Mll-AF9* fusion gene, despite the widespread activity of the *Mll* promoter.[300] These experiments confirm the use of homologous recombination strategies in investigating the role of oncogenic fusion proteins in leukaemogenesis. Recently, the fusion oncogenes *CBFA2-MTG8* and *CBFβ-MYHII*, have been recreated in ES cells by homologous recombination (see sections 1.4.1, 1.4.3).[225; 249]

1.9. Aim of Project

The previous finding that an artificially introduced oncogenic fusion gene contributed to leukaemogenesis proved that homologous recombination was an exciting new method for investigating the role of fusion oncogenes in tumourogenesis.[300]

Homologous recombination strategies have recently been used to investigate the role two fusion oncogenes, each one containing a sub-unit of the CBF complex, play in the development of leukaemia.[225; 249] Both studies implied unique functions of the oncogenic fusion products over and above the interruption of function of the CBF complex.

The reciprocal translocation in which the *ETV6* gene on chromosome 12 and the *CBFA2* gene on chromosome 22 are joined is the most common childhood genetic abnormality. As has been previously stated, both the *CBFA2* and *ETV6* genes are involved in numerous reciprocal translocations. The aim of this project was to generate an artificial fusion construct containing the human *ETV6-CBFA2* gene which could be introduced into the mouse *Etv6* gene locus by homologous recombination in mouse ES cells thus mimicking the t(12;21)(p12;q21).

Once the t(12;21) targeting construct had been successfully introduced into mouse ES cells, chimeric animals would be analysed to investigate the contribution of the fusion protein to haematopoiesis. Germline transmission of the fusion construct would then be achieved and it's involvement in definitive haematopoiesis and embryonic development assessed in heterozygous and homozygous embryos.

MATERIALS AND METHODS

2.1. Materials (see Appendix I)

2.2. Methods

2.2.1. DNA Preparation

2.2.1.1. Plasmid DNA (Minipreps)[229]

A cocktail stick was used to inoculate 1.5ml aliquots of 2x TY plus ampicillin (200μg/ml) with a single bacterial colony containing the desired plasmid constructs and incubated at 37°C overnight in a shaking incubator. The 1.5ml culture was transferred to an Eppendorf tube and spun at 13000 rpm in an MSE Microcentaur microfuge for 5 minutes to pellet the cells. Stocks were kept by streaking each cocktail stick on a 2x TY/ampicillin plate, which were then grown at 37°C overnight prior to storing, sealed, at 4°C.

Pellets were resuspended in ice cold GTE and left at room temperature for 5 minutes. 200μl of freshly prepared 0.2M NaOH/1% SDS was then added to lyse the cells. The tubes were rapidly inverted and placed on ice for 10 minutes. The bacterial DNA was precipitated by adding 250μl of 3M Na Acetate pH 4.8. The tubes were shaken and incubated on ice for at least 15 minutes. Spinning in a microfuge for 5 minutes to pellet the cell debris and *Escherichia coli* DNA. The supernatant was transferred to a fresh tube and the plasmid DNA was precipitated with 2 volumes of 100% cold ethanol. The tubes were left at -20°C for 10 minutes to maximise the amount of plasmid DNA precipitated. The DNA was then pelleted by centrifugation. The pellet was washed with 70% ethanol, dried in a vacuum dessicator for 5-7 minutes and resuspended in 30-50μl of TE. 5μl was analysed by restriction endonuclease digestion with the addition of 0.5μl DNAase free RNAase (10mg/ml). The plasmid inserts were then analysed by gel electrophoresis and visualised by ethidium bromide staining on a UV light transilluminator.

Alternatively, DNA was prepared from 1.5ml culture using the Quiagen miniprep kit, following the manufacturer's instructions.

2.2.1.2. Large Scale Plasmid Preparation (Maxi Prep)

100ml of 2xTY with 100μg/ml ampicillin was inoculated with a single bacterial colony containing the plasmid of interest and grown overnight in a shaking incubator at 37°C. 600μl of the overnight culture was used to prepare a 15% glycerol stock which was stored at -70°C. The remainder of the overnight culture was transferred to two 50ml Falcon tubes and spun for 15 minutes at 3000 rpm in a MSE Mistral 2000 centrifuge. 10ml of cold GTE was added to the pelleted cells in each tube, and incubated at room temperature for 10 minutes. The cells were then lysed by adding 20ml/per tube of freshly made 0.2M NaOH/1% SDS to each tube. The contents of the tubes were thoroughly mixed and incubated at room temperature for 5 minutes. The *Escherichia coli* DNA was precipitated by adding 10ml of 3M Na Acetate pH 4.8. Each tube was shaken vigorously before placing on ice for 15-60 minutes. The tubes were spun at 3000 rpm as before and the supernatant strained through gauze into fresh Falcon tubes. DNA was precipitated by the addition of an equal volume of isopropanol. After incubating on ice for 10 minutes the DNA was pelleted by centrifugation at 3000 rpm as before. The pellet was dissolved in 2ml/tube TE and 2ml/tube of 5M LiCl were then added to precipitate high molecular weight RNA. After incubating on ice for 5 minutes the tubes were spun at 3000 rpm again and the supernatant transferred to a fresh Falcon tube. 2 volumes of ice cold 100% ethanol were added to re-precipitate the DNA. The pellet was collected by centrifugation at 3000 rpm after incubating on ice for 5 minutes. The pellet was resuspended in 1ml/tube of TE and incubated at 37°C for 30 minutes with 4μl of 10mg/ml DNAase free RNAase, to remove the *Escherichia coli* RNA.

0.5ml of 2.5M NaCl/20% PEG was added, the reaction mix transferred to 1.5ml Eppendorf tubes, and the tubes incubated on ice for 10 minutes to collect the plasmid DNA. The DNA was pelleted at maximum speed in a MSE Microcentaur microfuge for 5 minutes. All excess PEG was removed and the pellets resuspended in 500μl TE. 2 PCIA extractions and a chloroform extraction were performed. The plasmid DNA was precipitated with 2 volumes of 100% ethanol and 10% 3M Na Acetate at -20°C for 20 minutes. The pellet was collected by centrifugation, washed once in 70% ethanol and dried in a vacuum dessicator for 5-10 minutes. The pellet was resuspended in 100-200μl of TE depending on the size of the pellet and the yield was determined by spectrophotometer at OD_{260}nm.

2.2.1.3. Preparation of Phage Vector High Titre Stock (HTS).

A single colony of *Escherichia coli* Q358[311] ells was transferred into a 5ml culture LB containing 50µl of 1 M $MgSO_4$ and 50µl 20% maltose and grown overnight at 37°C in a shaking incubator. The following day, 300µl of culture was aliquoted into each of 4, 5ml glass tubes containing 30µl maltose and 30µl $MgSO_4$. 5µl, 1µl, and 0.5µl of phage were then added to each tube. The tubes were incubated at 37°C for 15 minutes to allow the phage to attach to the cells and 3ml of top LB agar at 50°C was added to each tube and plated onto LB agar plates. The plates were incubated at RT for 10 minutes and at 37°C overnight. 5ml of λ diluent were then added to the plates showing most lysis and the plates sealed and stored at 4°C for 24 hours to allow the diluent to absorb all the phage. After this time the λ diluent was collected from the plates and stored at 4°C prior to use.

Alternatively, HTS was obtained as follows. An individual plaque from a plate generated as above was transferred into a 1.5ml Eppendorf tube containing1ml diluent for several hours. The supernatant of the lysed plaque was then incubated overnight with 1:100 dilution of a fresh overnight culture of Q358. The following morning a full lysis was observed. The supernatant was then transferred into a 10ml tube, spun for 5 min at 2000 in a Mistral centrifuge and supernatant transferred to a new tube for storage. Approximately 1 ml (or 1:100) was used to prepare bacteriophage DNA as described below.

2.2.1.4. Large Scale λ Bacteriophage Preparation

A single colony of *Escherichia coli* Q358 cells was transferred into a 5ml culture of LB containing 50µl of 1M $MgSO_4$ and 50µl of 20% maltose and grown overnight at 37°C in a shaking incubator. 1ml of the overnight culture was added to 100ml LB, in a 250 ml flask, containing 1ml of bacteriophage high titre stock (HTS), 1ml 20% Maltose, 1ml $MgSO_4$, and incubated at 37°C for 6.5 hours in a shaking incubator. The culture was stopped when complete bacterial lysis was visually observed. 5.9g NaCl was added and the flask incubated on ice for 1.5 hours. The mixture was transferred into two 50ml Falcon tubes and spun in an MSE Mistral centrifuge at 4000 rpm for 20 minutes. 5g PEG 6000 was then added to the supernatant, allowed to dissolve, and incubated at 4°C for 1

hour or overnight. The Falcon tubes were spun at 4000 rpm as before, for 20 minutes, and the supernatant discarded. The remaining pellet was resuspended in 5ml λ diluent and extracted once with an equal volume of chloroform. The two tubes were combined and 1g of PEG 6000 was added, allowed to dissolve, and incubated on ice for 2 hours. The pellet was collected by centrifugation as before, resuspended in 400µl of λ diluent and transferred to a 1.5mlEppendorf tube. 10µl of 10mg/ml RNAase and 25µl of 1mg/ml of DNAase were added, in order to remove the unwanted RNA and DNA from the Q358 cells, and the preparation was incubated at 37°C for 30 minutes. To remove the enzymes, 500µl of chloroform was added and after mixing the aqueous phase was collected by centrifugation at 13000 rpm for 10 minutes in a MSE Microcentrifuge. The supernatant was transferred to a fresh tube and the coat of the phage was lysed by the adding 20µl of 5mg/ml proteinase K and 25µl of 10% SDS, incubated at room temperature for 5 minutes and at 65°C for 1 hour. One phenol and one PCIA extraction were performed and the phage DNA in the supernatant was precipitated with an equal volume of cold 100% ethanol containing 10% 3M Na acetate. The DNA was collected by centrifugation at 13000 rpm for 20 minutes and the pellet washed with 70% ethanol, air dried and resuspended in 400µl of TE. The yield was determined by spectrophotometer at OD_{260}nm and the DNA was stored at -20°C prior to use.

2.2.1.5. DNA Extraction from Tissue Samples

DNA was extracted from mouse tail samples and ES cells using the Purescript DNA isolation kit (Gentra Systems) as described below.

ES cells were resuspended in 150µl cell lysis solution and incubated at room temperature for 2 hours. The lysate was transferred to a 1.5ml Eppendorf tube and 50µl of protein precipitation solution was added. The mixture was incubated on ice for 15 minutes and spun at 13000 rpm in an MSE Microcentaur centrifuge for 10 minutes. The DNA was precipitated by adding 150µl of isopropanol and incubating at –20°C overnight. The DNA was pelleted by centrifugation at 13000 rpm for 20 minutes and the pellet washed once with 500µl of 70% ethanol. The DNA was dried in a vacuum dessicator and resuspended in 40µl TE.

Tail samples were placed in a 1.5ml Eppendorf tube containing 500µl cell lysis solution (containing proteinase K at a concentration of 1µg/µl) and incubated at room temperature overnight. The tubes were centrifuged at 13000 rpm for 5 minutes to remove any debris and the supernatant transferred to a fresh tube. 167µl of protein precipitation solution was added. The mixture was incubated on ice for 15 minutes and spun at 13000 for 10 minutes. Adding 150µl of isopropanol and incubating at –20°C overnight precipitated the DNA. The DNA was pelleted by centrifugation at 13000 rpm for 20 minutes and the pellet washed once with 500µl of 70% ethanol. The DNA was dried in a vacuum dessicator and resuspended in 60µl TE.

2.2.2. Restriction Endonuclease Digestion of DNA

For mapping and analysis of plasmid DNA with restriction endonucleases, 500ng-3µg of DNA were digested in a total volume of 30-50µl, with a 5-10 fold excess of enzyme. All digests were carried out in 1x Carlo's buffer, 1mM DTT and 3mM spermidine. The total volume of enzyme added did not exceed 10% of the total digest volume. Digests were incubated for a minimum of 3 hours at the appropriate temperature. For genomic DNA Southern blots, 10µg of DNA were digested in 50µl for at least 5 hours.

2.2.3. Agarose Gel Electrophoresis of DNA

All DNA samples were mixed with 0.2 volumes of loading buffer before electrophoresis. Restriction digests of plasmids and genomic DNA were resolved in 0.8% agarose gels containing 1µg/ml ethidium bromide. For the high molecular weight genomic DNA digests required for accurate mapping, 1.0x AGB gels (24.5cm) were run at 40 volts for 18-24 hours in a Flowgen tank. Other digests were run on 0.5x TBE gels (12.5cm) at 100 volts for 1-1.5 hours in a Hybaid tank. Small PCR products and other small digestion fragments were resolved on higher concentration agarose gels i.e. 1-2% gels. The size markers used were either λ bacteriophage cut with Hind III (NBL Gene Science) or ϕX 174 cut with Hae III (Gibco BRL).

2.2.4. Preparation of DNA Probes

Following restriction endonuclease digestion of plasmid clones containing fragments to be used as probes, the fragments were resolved on agarose gels. The fragment of interest was excised under UV light with a scalpel blade. DNA was purified from the agarose slice using a Jetsorb DNA extraction kit (Genomed) according to the manufacturer's instructions and the DNA was eluted in a final volume of 40µl TE. Alternatively, the agarose slice was placed into a 0.5ml Eppendorf tube on top of glass wool packed and a hole pierced through the bottom of the tube. The Eppendorf was placed into a 1.5ml Eppendorf and spun at 13000 for 30 seconds to collect the flow-through. DNA was then further purified using PCIA and a G50 column, prior to any further manipulation. OD was checked by spectophotometry.

2.2.5. Cloning of DNA into Plasmid or Phagemid Vectors

2.2.5.1. Vector Preparation

1µg of uncut vector e.g. Bluescript plasmid (Stratagene) or M13mp18 bacteriophage (Boehringer Mannheim) was digested in a 50µl digest with the appropriate restriction enzyme/s required. After 3 hours incubation at the appropriate temperature a 5µl aliquot electrophoresed on a 0.8% agarose gel with 50ng of uncut vector in parallel with a molecular weight marker, to check for complete digestion of the vector. The reaction was then placed at 65°C for 10 minutes to inactivate the enzymes before being placed briefly on ice. 2-4µl of calf intestinal alkaline phosphatase (Boehringer Mannheim 1U/µl) was added to dephosphorylate the vector ends and the reaction was incubated at 37°C for one hour. The reaction was diluted to 100µl with TE and mixed with 100µl PICA. The aqueous phase was collected by centrifugation and was centrifuged through a G50 Sephadex column equilibrated with 100µl TE, to remove residual phenol. The vector was stored at -20°C and 2µl (20ng) of vector was used for each ligation reaction.

2.2.5.2. Filling In Procedure

The vector was initially digested with the appropriate restriction enzyme as described before (2.2.2.1). Once the vector had been fully digested, the reaction was heat

inactivated at 65°C for 10 minutes. The reaction was diluted to 100µl with TE and was centrifuged through a G50 Sephadex column equilibrated with 100µl TE. Filling in was then carried out as follows:

19µl	cleaned, digested plasmid DNA
3µl	dNTP's (0,5mM)
3µl	Carlos' buffer
3µl	DTT (15mM)
2µl	T4 DNA polymerase enzyme (New England BioLabs 3U/µl)

Tubes were incubated at 37°C for 30 minutes and heat inactivated at 65°C for a further 10 minutes. 5.0µl of DNA was added to a ligation reaction and incubated at 14°C overnight (2.2.7.4). 8µl of ligation reaction was then incubated with 200µl of competent cells and plated out (2.2.8.1). Colonies were picked and miniprep DNA produced. The DNA was digested with the appropriate enzyme to ensure removal of the site. Maxiprep DNA was then made from a suitable clone.

2.2.5.3. Insert Preparation

These methods are for preparing inserts with overhanging ends. For preparation and cloning of blunt ended fragments, see 2.2.2.4. Depending on the size of the fragment to be cloned, 5-20µg of DNA was digested with 5-10 fold excess of appropriate restriction enzyme under optimal conditions. The digested products were run on an ethidium bromide agarose gel of a concentration appropriate to separate the fragment of interest. The band to be cloned was excised using a scalpel blade under UV light and the DNA purified using a Jetsorb kit and eluted into 40µl TE, following the manufacturer's instructions.

2.2.5.4. Ligation

Two ligation reactions were set up for each insert, usually with a 1-3 molar excess of insert DNA and 20ng (2µl) of vector, plus a control ligation with vector only. Ligations were carried out in a total volume of 20µl using 200-400U of T4 DNA ligase New England Biolabs (400U/µl)) in 1x ligase buffer with 1M ATP (New England Biolabs). 2µl of vector (approximately 40ng) was used for each reaction. The ligations were incubated at 14°C overnight. The ligations were then stored at -20°C until required.

2.2.5.5. Sonication of DNA and Shotgun Cloning into M13mp18

Some inserts were too long to sequence from the Bluescript vector directly, and it was necessary to break the DNA up into smaller pieces and subclone it into another vector e.g. M13mp18 for sequencing. Sonication of DNA was used to achieve this.

The plasmid DNA to be sequenced was prepared by the plasmid maxiprep method (2.2.1.3). 15µg of DNA was diluted up to 100µl with TE in a 500µl Eppendorf tube. This was then placed in a sonicator (Ultrasonic Processor) and sonicated on full setting for 40 seconds before placing on ice for 1 minute. This cycle was repeated 5 times. The size of the fragments was estimated by running 5µl of the sonicated DNA in a 1.5% agarose gel with ϕX 174-Hae III marker. If the fragments were larger than 600bp then further cycles of sonication were performed and the sample re-analysed by electrophoresis.

The ends of the fragmented DNA were filled in prior to cloning. 15µl of the sonicated DNA was added to the following:

3µl 10mM dNTPs

3µl 10x Carlo's buffer

3µl 15mM DTT

2µl Klenow (sequencing grade Boerhinger Mannheim 5U/µl)

4µl distilled water

The reaction was incubated at 37°C for 30 minutes before heat inactivation of the Klenow at 65°C for 10 minutes. The sonicated DNA was then ligated into an M13mp18 vector prepared by digestion with the restriction enzyme Sma I at 25°C for 5 hours. The following day the ligations were transfected into competent cells. Positive colonies for sequencing were identified by hybridisaton to the original plasmid insert DNA that had been radiolabelled (2.2.1.7). By sequencing a series of smaller clones the full sequence of the larger fragment was assembled using DNAStar software.

2.2.6. Transformation of Bacterial Cells with Recombinant Clones

2.2.6.1. Preparation of Hannahan's Competent Cells[312]

A single colony of the TG1 strain of *Escherichia coli* cells[311] was inoculated in 5ml of SOB medium containing 50µl of 20% glucose and 50µl of 1M $MgCl_2$. This was then grown overnight in a shaking incubator at 37°C. 500µl of the overnight culture was diluted in 50ml of SOB medium in a sterile, 250ml flask. 500µl of 20% glucose and 500µl of 1M $MgCl_2$ was also added and the flask was returned to the 37°C incubator. The culture was then grown to an OD_{600}nm of 0.5-0.55. The cells were placed on ice for 15 minutes and then the culture was transferred to a 50ml Falcon tube. The cells were pelleted by centrifugation at 1600 rpm for 10 minutes. The cell pellet was resuspended in $1/3^{rd}$ of the original culture volume (16.5ml) of cold TFB and left on ice for 10 minutes. The cells were then spun as before and the pellet resuspended in $1/12^{th}$ of the original culture volume (4ml) of TFB. DnD was then added to 7% of the final TFB volume, in 2x3.5% aliquots. The cells were incubated on ice for 10 minutes before a second DnD aliquot was added. After a further 15 minutes incubation on ice the cells were ready for transfection.

2.2.6.2. Transfection of Competent Cells

200µl of competent cells were mixed with 8µl of ligation in a 1.5ml Eppendorf, incubated on ice for 45 minutes and then heat shocked in a 42°C water bath for 2 minutes.

For Bluescript transfections, 400µl of 2x TY were added and the cells were incubated at 37°C for 30 minutes to allow expression of the ampicillin resistance gene to occur prior to plating. The whole culture was then plated onto 2x TY agar plates supplemented with ampicillin (200µg/ml). For M13 ligations, the transfected cells were transferred to a 5ml glass tube prior to adding 3ml of 2x TY top agar at 50°C. The cells plus agar were quickly poured onto 2x TY agar plates. The plates were then incubated overnight at 37°C.

2.2.7. Southern Blotting[313]

After agarose gel electrophoresis of DNA (2.2.3), a photograph of the gel was taken and the size markers highlighted with ink using a 19-gauge needle. The gel was then placed in depurination solution and gently agitated for 20 minutes. Depurination of sites within DNA allows cleavage during denaturation, fragmenting the longer pieces of DNA and enabling more efficient transfer to the membrane of the high molecular weight DNA. This step was omitted if all fragment sizes were below 10kb. The gel was then rinsed in distilled water before soaking in denaturing solution for 30 minutes. The gel was then transferred to neutralising solution for a further 30 minutes, following a quick rinse in DDW to remove dentauration solution. The gel was then capillary blotted onto Hybond nylon filters (Amersham Life Sciences) in a transfer tank containing 20x SSC overnight. Small gels containing digested plasmid DNA were dry transferred on the bench for 1 hour, or overnight. The DNA was fixed to the filters by UV fixation (Stratalinker) and/or baking at 80°C in a vacuum oven for 2 hours. Filters were rinsed in 3x SSC prior to hybridisation.2µl of probe DNA (approximately 40-80ng) was diluted to 48µl with distilled water and boiled for 5 minutes before placing on ice. The 48µl was then collected by brief centrifugation and added to a pre-prepared Rediprime (random prime labelling, Amersham Life Sciences) tube for labelling DNA. 2µl of [$\alpha - ^{32}$P]dCTP (10µCi/µl) was added and the reaction was incubated at 37°C for 1 hour. The reaction was diluted with water to 100µl and unincorporated nucleotides were separated from the DNA probe by centrifugation through a G50 Sephadex column. This was prepared in a 1ml syringe plugged with glass wool and equilibrated with distilled water. The labelled

probe was boiled for 5 minutes to denature the probe before being added directly to the hybridisation solution.

2.2.8. *In Situ* Colony Hybridisation[314]

In order to select recombinants containing the desired insert, plaques or colonies were screened by in situ hybridisation as follows. Lifts were taken from each plate onto Whatman 1MM paper filters. The filters were laid over the plate surface for 1-2 minutes. When the paper became wet, orientation marks were made using a 19-gauge needle and black ink. The filters were then carefully removed from the plates. For M13 transfections the DNA was fixed to the filters by UV fixation and used directly.

For Bluescript colonies, the filters were placed colony side up, on 3MM Whatman paper soaked in 2x SSC/5% SDS for 2 minutes to lyse the cells. DNA was then fixed to the filters by microwaving on the highest setting for 2.5 minute. After rinsing the filters in 3x SSC, the filters were ready for prehybridisation.

2.2.9. Filter Hybridisation

Filters were pre-hybridised at 65°C for 1-2 hours in Church buffer (Dr T Rabbitts, personal communication). Labelled probe was then added to the hybridisation solution and incubated with the filters. Genomic Southern blots were hybridised overnight. Filter lifts of plasmid colonies were hybridised for only 4 hours.

The stringency of the washes depended on the hybridisation that had been performed. Normally filters were washed in 3x SSC/0.1% SDS initially at room temperature for 10 minutes and then for a further 10 minutes at 65°C. Further washes for 20 minutes at 65°C in 1x SSC/0.1% SDS and 0.5x SSC/0.1% SDS were performed often performed. If, following exposure, filters displayed excess background radiation then they would be washed in more stringent conditions and re-exposed.

Filters were exposed to Fuji Medical X-ray film (RX) at -70°C. The time of exposure varied from 2 hours to 14 days depending on the intensity of the signal. Positive hybridisation signals could be identified using orientation marks and the size of hybridisation bands were assessed by comparison to marked DNA molecular markers.

2.2.10. Manual Sequencing of Single-Stranded Plasmid DNA

2.2.10.1. Single-Stranded Plasmid DNA Preparation (ssDNA)

Colonies of interest for sequencing of plasmid or phage DNA were picked using a sterile cocktail stick into a glass tube containing 1.5ml of 2x TY. If plasmid DNA was to be rescued, ampicillin at a concentration of 200μg/ml was added. These cultures were then incubated at 37°C in a shaking incubator overnight.15μl of the overnight culture was then transferred to the new tubes containing 1.5ml of 2x TY (plus antibiotic if required). The tubes were then returned to the 37°C incubator. The plasmid cultures were grown for 2 hours and then 3μl of Helper Phage (VCS-M13, Stratagene) was added to each tube to rescue the single stranded copy of the DNA. The cultures were then incubated for another 5.5 hours at 37°C with vigorous shaking.

For M13 clones, an overnight TG1 culture was diluted to 1: 100 into 2TY medium and with the help of a toothpick a plaque was transferred into the tube and then the clture was grown for 5.5 hrs in a shaker at 37°C. DNA was collected as decribed below.

After incubation, both the M13 and plasmid cultures were transferred to 1.5ml Eppendorf tubes and spun for 5 minutes at maximum speed in a MSE Microcentaur microfuge. The supernatent was transferred to a fresh tube containing 200μl of 20% PEG/2.5M NaCl. The tubes were then left at room temperature for a minimum of 10 minutes before spinning again at maximum speed for 5 minutes. After the supernatant was discarded, the pellet was carefully dissolved in 100μl TE and 200μl of PCIA was added .The mixture was vortexed for 5 seconds before spinning at 13000 rpm for 5 minutes to collect the aqueous phase. The aqueous phase was transferred to a fresh tube and the previous step was repeated. 100μl of chloroform/isoamyl alcohol (24:1) was then added to the aqueous phase to remove any residual phenol. The two layers were mixed by vortexing and the tubes spun again. The aqueous phase was transferred to a new tube and 10% of a 3M Na Acetate and 250μl 100% ethanol were added to precipitate the single stranded DNA (ssDNA). The DNA was incubated on ice for at least 20 minutes before being recovered by centrifugation at 13000 rpm for 5 minutes. Alternatively, the DNA was precipitate by incubating the tube at –70°C for 2 hrs or overnight at –20^{0}C. The pellet

was washed with 70% ethanol and then air-dried. Each pellet was then resuspended in 30µl TE and stored at -20°C until required.

2.2.10.2. Manual DNA Sequencing

The dideoxy chain termination method for manual sequencing was originally described by Sanger *et al.*[301] All sequencing reactions were performed in U bottom microtitre plates. 2µl of ssDNA from each clone was transferred into 4 vertical wells. 2µl of the following master mix were then added to each well; 1µl reaction buffer, 1µl -40 primer and 6µl water. The plates were then spun briefly in a MSE Mistral 2000 centrifuge to collect the reaction mix at the bottom of the well, covered in Saran wrap and incubated at 55°C for 30 minutes. During this time a master mix was made up for each clone as follows:

0.4µl 7.5mM dNTP

0.4µl 100mM DTT

6.5µl water

0.5µl ^{35}S-dATP (Amersham Life Sciences)

0.25µl sequenase enzyme (Amersham Life Sciences 13U/ml))

After incubation at 55°C 2µl of the above solution was transferred to each well. The plates were spun again and incubated at room temperature for 10 minutes. 2µl of one of four stop nucleotide mixes (Amersham Life Sciences 250µl) i.e. T, C, G or A were added to each of the four wells. The plates were briefly spun as before and incubated at 37°C for 6 minutes. 4µl of formamide stop solution was added to each well to terminate the reaction. Prior to loading the sequencing gel the reactions were incubated at 80°C for 20 minutes.

M13 –40 primer sequence 5'-GTTTTCCCAGTCACGAC-3'

2.2.10.3. Acrylamide Gel Electrophoresis for DNA Sequencing

Sequencing electrophoresis was performed using 40x50x0.4cm gradient gels. Before pouring the gel, the plates were cleaned and both plates were then siliconised with Repelcote. The plates were separated by 0.4mm spacers and fastened together with tape and bulldog clips. 30µl of 10% ammonium persulphate solution (APS) and 13µl of Temed (Amresco) was added to 15ml of 2.5x TBE acrylamide gel mix and the reaction was poured into the plates using a 50ml syringe. This was quickly followed by the second mix of 50ml 0.5x TBE acrylamide gel mix, 100µl APS and 65µl Temed. A comb to form the wells was placed in position and the gel was allowed to set for 30 minutes. The tape and bulldog clips were removed and the gel was then placed in Gibco BRL S2 gel tank, 1 litre of 0.5x TBE was used as buffer and 4µl of each sample reaction was loaded into each well. The gel was run at 30 volts for 3.5-4 hours. The plates were then disassembled and the back plate removed. The acrylamide gel was transferred to 3MM Whatman paper and covered in Saran wrap. Before being dried under vacuum at 80^{o}C on a gel dryer for 2 hours. The dried gel was then exposed overnight at room temperature to Fuji Medical X-ray film (RX). Sequences were analysed using DNAStar software.

2.2.11. Automated Sequencing of Double Stranded Plasmid DNA

This technology allows direct sequencing of a plasmid insert from double stranded DNA. The machine used for the majority of the project was an ABI Prism 377 (Perkin Elmer). In this system the DNA template was labelled with four fluorescent dyes, one for each nucleotide, using a PCR primer extension method. The fragments were then electrophoresed through a denaturing acrylamide gel to size separate the fragments. At the base of the gel a laser beam continuously scans the gel, exciting the fluorescent dyes attached to each fragment. The dyes emit light at a specific wavelength upon excitation and is collected and separated by a spectograph onto a cooled, charged couple device. The computer software then collects these data and stores them for processing as readable sequence.

2.2.11.1. Reactions for Automated Sequencing

All DNA was prepared either by maxiprep or by miniprep using the Qiagen spin column miniprep kit as already described (2.2.1.1, 2.2.1.2). The DNA was quantitated by

spectrophotometry at OD_{260}nm and 400-600ng was used for each reaction. The DNA was placed in a 250ml PCR tube and the volume adjusted to 8µl with sterile water.

The following was then added to each tube:

1µl Big Dye (Perkin Elmer)

7µl 2.5x automated sequencing buffer

4µl primer at 0.8pmol/µl

The Big Dye contains a set of 4 fluorescently labelled dye terminators as well as AmpliTaq DNA Polymerase.

The contents of the tube were gently mixed before being placed in a Perkin Elmer Gene Amp PCR thermocycler. The following PCR profile was used for all sequencing reactions:

25 cycles at	96°C	10 seconds
	50°C	5 seconds
	60°C	4 minutes

The DNA was precipitated by transferring the reaction mix to a 500µl tube containing 50µl 100% ethanol and 2µl 3M Na acetate pH 4.8 and incubating on ice for 1 hour. The DNA was pelleted by centrifugation at 13000 rpm in a MSE Microcentaur microfuge for 30 minutes. The pellet was washed in 300µl 70% ethanol, briefly vortexed and re-collected by spinning at 13000rpm for a further 5 minutes. The DNA pellet was vacuum dried for 5 minutes before being resuspended in 8µl formamide loading buffer (5 parts deionised formamide-Amresco, and 1 part loading dye-Perkin Elmer).

Just prior to loading the sequencing gel, the DNA was denatured by incubation at 98°C for 2 minutes. The denatured DNA was kept on ice until loading

2.2.11.2. Electrophoresis Using the ABI 377 Automated Sequencer

All methods used for the ABI 377 sequencer were in accordance with the manufacturers instructions. Perkin Elmer supplied all the apparatus. The plates were assembled with 0.2mm spacers. The gel mix was made up with 18g urea dissolved in

25ml double distilled water. 0.5g of mixed bed ion exchange resin and 5ml of acrylamide-bisacrylamide (40%(w/v) (19:1) solution, Kramel Biotech) was then added and the solution was vacuum filtered to remove the resin and to degas the gel mix. 5ml of filtered 10x TBE was then added and the mix made up to a final volume of 50ml with double distilled water. Prior to pouring, 250µl of 10% APS and 35µl of Temed (Amresco) were added. The gel was poured using the supplied gel pouring apparatus and allowed to set for 2 hours prior to use.

2µl of each sample was run per well and electrophoresis was carried out for 7 hours.

All generated sequence was analysed using DNAStar software.

2.2.12. RNA Extraction

RNA was extracted from ES cells and cell lines using the Purescipt RNA isolation kit (Gentra Systems). Cells were collected into a 1.5ml sterile Eppendorf tube and pelleted by centrifugation at 13000 rpm for 5 minutes. The pelleted cells were lysed by adding 500µl cell lysis solution and incubating at room temperature for 2 hours. 167µl protein precipitation solution was added and the tubes were incubated on ice for 15 minutes. The tubes were spun at 13000 rpm for 10 minutes and the supernatant transferred to a fresh, sterile Eppendorf tube. Adding 150µl of isopropanol and incubating at –20°C overnight precipitated the RNA. The RNA was pelleted by centrifugation at 13000 rpm for 20 minutes and the pellet washed once with 500µl of 70% ethanol in DEPC water. The RNA was dried in a vacuum dessicator and resuspended in 10µl DEPC water and stored at -70 prior to use.

Alternatively, RNA was recovered from cell cultures using the Midi-Rnaesy preparation kit from Qiagen following the manufacturer's instructions.

2.2.13. cDNA Preparation by Reverse Transcriptase

To make cDNA, 5µl of RNA were transferred into a sterile 500µl Eppendorf tube and 1µl of RNAsin (Promega 200U/µl) was added. The volume was adjusted to 20µl with DEPC water and the tube incubated at 65°C for 5 minutes before being cooled on ice for a further 5 minutes. The following were then added:

1µl	RNAsin (Promega 200U/µl)
10µl	5x reverse transcriptase buffer (Gibco BRL)
5µl	5mM dNTPs
0.5µl	0.1M DTT (Gibco BRL)
1µl	random hexamer primers (Pharmacia 0.5µg/µl)
1µl	MMLV reverse transcriptase (Gibco BRL 200U/µl)
	DEPC water to a final volume of 50µl

The reaction was incubated at 37°C for 1 hour and then heat inactivated at 65°C for 10 minutes. 2µl of cDNA are then used for each subsequent PCR reaction.

2.2.14. Polymerase Chain Reaction (PCR)[315]

All PCR reactions were carried out in 50µl volumes containing 1x PCR buffer (Promega) containing $MgCl_2$ to a final working concentration of 1.5mM, 0.5µl Taq DNA Polymerase (Promega 5U/µl), 250ng of each primer, and 5µl 2mM dNTPs. The volume was then made up with sterile water and DNA template. A drop of mineral oil (Sigma) overlaid each reaction to prevent evaporation.

A standard program was used for all PCR reactions with the annealing temperature being dependent on the Tm of the primers used.

Hot start	94°C	5 minutes	1 cycle
Denaturation	94°C	1 minute	
Annealing	X°C	1 minute	
Extension	72°C	2 minutes	35 cycles
Extension	72°C	10 minutes	1 cycle

PCR products were characterised by electrophoresis on agarose gels containing 1µg/ml ethidium bromide. The concentration of the gel depended on the expected size of the PCR product to be resolved.

Alternatively, RT-PCR was performed using the OneStep RT-PCR kit (Qiagen) according to the manufacturers instructions.

2.2.15. Site-Directed Mutagenesis (Stratagene)

Site directed mutagenesis is a technique by which single or multiple bases in a DNA sequence can be altered, deleted or added.

The method was performed on double stranded template DNA in Bluescript plasmid.

Sample preparation

	5µl	10x reaction buffer (Stratagene)
	5µl	1or10ng/µl dsDNA template
	12.5µl	forward oligonucleotide primer (10ng/µl)
	12.5µl	reverse oligonucleotide primer (10ng/µl)
	1µl	10mM dNTP mix
		make up to 49µl with double distilled water
Add	1µl	native Pfu DNA polymerase (2.5U/µl Stratagene)

Overlay each reaction with 1 drop of mineral oil (Sigma)

Cycling reactions

Each reaction was placed in a thermo cycler (Hybaid) and cycled according to the following parameters:

Segment	Cycles	Temp.	Time
1	1	95°C	30 seconds
2	10-18	95°C	30 seconds
		55°C	1 minute
		68°C	2 minutes/kb of plasmid length

Segment 2 parameters were adjusted in accordance with the type of mutation required:

Point mutations	12 cycles
Single amino acid changes	16 cycles
Multiple amino acid deletions or insertions	18 cycles

Following thermo-cycling 1μl of Dpn I restriction enzyme (10U/μl) was added directly to each amplification reaction below the mineral oil overlay. Each tube was mixed and incubated at 37°C for 1 hour.

Dpn I restriction enzyme (target sequence 5' -G^{m6}ATC- 3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesised DNA. DNA isolated from most *Escherichia coli* strains, including TG1, is dam methylated and therefor susceptible to Dpn I digestion.

1μl and 10μl aliquots were then used to transfect competent cells.

2.2.16. Routine Tissue Culture

The REH (ATCC CRL-8286) and EL4 (ATCC number CRL-8286) cell lines were obtained from the American Type Culture Collection, Rockville, Maryland, USA. Frozen cell lines were thawed rapidly in a 37°C water bath, added to 20ml Hanks' Balanced Salts Solution (HBSS, Gibco BRL) in a 50ml Falcon tube and pelleted by spinning at 1200 rpm in an MSE Mistral 2000 centrifuge. On removal of the supernatant the cells were washed once with 20ml HBSS before being resuspended in 40ml of RPMI 1640 medium (Gibco BRL) containing 10% Foetal Calf Serum (FCS, Gibco BRL), 2mM L-Glutamine (Gibco BRL) and 50μg/ml Penicillin-Stroptomycin (Gibco BRL). Both cell lines were grown at 37°C in tissue culture flasks in a 5% CO_2 incubator. Cells were maintained in logarithmic phase of growth, with regular freezing of aliquots. For freezing, 5-10 x 10^6 cells were taken from a healthy culture and spun in a MSE Mistral 2000 centrifuge at 1200 rpm for 5 minutes. The pelleted cells were resuspended in 1ml of freezing medium (50% FCS, 10% DMSO, 40% HBSS). Cells were aliquoted into 2ml

freezing files, transferred to a -70°C freezer for 24-48 hours and then stored in liquid nitrogen.

2.2.17. Embryonic Stem Cells

All ES cell work was performed by Richard Pannell in Dr Terry Rabbitts' laboratory in the Medical Research Council Laboratory of Molecular Biology, Cambridge unless otherwise stated.

2.2.17.1. Routine Culture

ES cells were cultured according to the published guidelines [302] using batch tested foetal calf and newborn calf serum. For 500ml ES medium; 400ml of DMEM GPS was supplemented with 50ml foetal calf serum (FCS), 50ml newborn calf serum (NCS), 5ml non-essential amino acids, 5ml nucleosides stock (to 100ml sterile DDW, add 80mg adenosine, 85mg guanosine, 75mg cytidine, 73mg uridine and 24mg thymidine dissolved by heating to 37°C), 5ml β-mercaptoethanol stock (to 10ml sterile PBS add 7μl of β-mercaptoethanol), and 5ml CHO-LIF supernatant. The complete medium was then filtered through 0.45μm Nalgene.

2x ES cell freezing medium consisted of 60% DMEM, 20% DMSO, 10% NCS and 10% FCS.

ES cells were trypsinised in a single well of a 24 well plate by adding 200μl of ES trypsin, incubating at 37°C for 3 minutes, then neutralising the trypsin with 1ml ES cell medium. The cells were dispersed using a 1ml pipette, and 0.5ml of this mix was added to an equal volume of 2x freezing medium in a 2ml Nunc freezing vial. To the remainder of the cell suspension, 1ml of ES cell medium was added in the tissue culture well to allow expansion of ES cells for harvesting of DNA. The freezing vials were transferred to -20°C overnight, then placed in -70°C for at least 24 hours prior to long term storage in liquid nitrogen. ES cells were rapidly thawed by placing the vial at 37°C. The cells were transferred to 5ml of complete ES cell medium in a 15ml Falcon tube, spun at 1000 rpm for 5 minutes, resuspended in 2ml of ES cell medium, and added to feeder cells in a single well of a 24 well plate. ES medium was changed daily to prevent acidification. Cells were split every 2-3 days at 1:6 to 1:8.

2.2.17.2. STO Cells

G418-resistant STO fibroblasts were made by transfecting the plasmid pRSVneo into STO cells. A. J. H. Smith at the LMB in Cambridge derived this line which was maintained in DMEM GPS + 10% FCS. STO fibroblasts were mitotically inactivated for use as a feeder layer using the mitomycin C method detailed in 2.2.17.3.

2.2.17.3. Preparation of Feeder Cells

G418-resistant STO feeder cells were prepared from confluent 150 cm^2 tissue culture flasks by aspirating the growth medium and replacing with 10ml of DMEM + 10% FCS + 10μg/ml of mitomycin C (2mg mitomycin C was pre-dissolved in 1ml sterile PBS). The flasks were incubated for a minimum of 2 hours at 37°C, the medium aspirated and the cells washed twice in sterile PBS. Mitotically inactivated feeder cells were then trypsinised, collected in DMEM + 10% FCS, counted and frozen down at 10^7 cells per vial. On thawing, the feeder cells were resuspended to give a total of 2x 96 well plates, 2x 24 well plates, 2x 6 well plates or 2x 10cm Petri dishes. The dishes had been gelatinised for at least one hour by covering in a 0.1% gelatin solution.

2.2.17.4. Preparationof Supernatant From CHO 8/24 720 LIF-D(1)

The cell line was a gift from the Genetics Institute, MA, USA. Derived from Chinese Hamster Ovary, they were cultured in alpha-MEM (Sigma) supplemented with 10% heat inactivated FCS, 1mM L-Glutamine, 1% Pen-Strep, and 0.1μM Methotrexate (Sigma). Cells were thawed and plated onto 150cm^2 tissue culture flasks. The medium was changed after attachment of the cells, and on day 3 they were split 1:10. When these cells were 50% confluent, they were washed 3 times in sterile PBS, and 20ml ES cell medium added. 2-3 days later, the supernatant was collected, centrifuged at 2000 rpm for 10 minutes, filtered through Nalgene 0.2μm, and aliquots stored at -20°C. LIF supernatant was added to ES cell medium at a dilution of 1:100.

2.2.17.5. Transfection of ES Cells

ES cells were fed 3 hours prior to trypsinisation, resuspended in DMEM GPS (no serum), counted, pelleted and 10^7 cells resuspended in 0.3ml of HBSS/β mercaptoethanol

buffer (10^{-4}M β-mercaptoethanol). 0.3 ml of suspension was added to 12.5 or 25μg of linearised DNA (at 2.5μg/μl) in a Falcon tube, mixed, and left at room temperature for 10 minutes. The suspension was then added to a 0.4cm cuvette (Biorad) and electroporation performed at 400V, 25μF in a Biorad Gene Pulser. Time constants of 1msec were recorded. After a further 10 minutes at room temperature, cells were plated onto 10cm Petri dishes with feeders. After 36 hours incubation at 37°C, cells were trypsinised and re-plated into selective medium (containing G418 at 400μg/ml and FIAU at 1μM) at 5 x 10^6 per 10cm Petri dish. Between days 8-10 following the re-plating, single colonies were picked using a 200μl pipette and a yellow tip into a single well of a 96 well plate containing 10μl sterile PBS. 40μl of trypsin was added and the plates incubated at 37°C for 7 minutes. After checking the wells to confirm disaggregation, 150μl of complete ES cell medium was added and the contents of each well transferred to a single well of a 96 well plate containing feeder cells. These cells were allowed to re-grow, trypsinised using a volume of 25μl and the trypsin inactivated by adding 75μl of complete ES medium. 50μl of this mix was added to a single well of a gelatinised 96 well plate and allowed to re-grow by adding a further 150μl of ES medium to provide enough cells for DNA analysis. If greater numbers of cells were needed the mix was re-plated into single wells of a 24 well plate. To the remaining 50μl of cell mix was added an equal volume of 2x freezing solution and the plate transferred to -70°C.

2.2.17.6. Karyotyping of ES Cells

This procedure was used to determine the chromosomal complement and banding pattern of the ES cell lines. Cells from a single well of a six well plate were used. The medium was removed and the cells washed twice with PBS prior to being trypsinised for 3 minutes. The cells were resuspended in the previously removed medium to inactivate the trypsin and centrifuged at 1200 rpm for 10 minutes. The medium was removed with a pipette and the cells were resuspended in 5ml 0.075M KCl (stored at 37°C), mixed well and incubated at 37°C for 15 minutes. The cells were again centrifuged at 1200 rpm for 10 minutes, the supernatant removed leaving a little to resuspend the pellet, and 1ml cold fix solution (3 parts methanol: 1 part acetic acid) added dropwise. A further 4ml of fix solution was then added. The cells were spun at 1200 rpm for 5 minutes, the supernatant removed and the cells resuspended in 5ml of fix solution. This was then repeated once

more and the cells resuspended in fix solution at a cloudy consistency. 2 drops of the cell suspension were added to a clean microscope slide and allowed to dry. The slides were placed on a hot plate for 1 hour and then left at room temperature for a further day. Slides were quickly dipped in a coplin jar containing 1-2% trypsin in PBS. Slides were then washed with PBS and then with pH 6.8 buffer solution ("Gurr" Gibco BRL). Slides were then placed on a staining rack and flooded with 20% Leishman staining solution in pH 6.8 buffer for 90 seconds. Slides were washed with tap water, dried on a hot plate and mounted with a cover slip prior to examination.

RESULTS

The aim of the experiments described in this chapter were to generate an artificial fusion gene in which a human *ETV6-CBFA2* construct was cloned, in frame, into the mouse *Etv6* gene locus, thus mimicking the t(12;21)(p12;q21). The targeting construct was also designed to allow for the correct processing of the transcribed DNA, and to allow for positive and negative selection. The targeting construct could then be introduced into the murine *Etv6* locus by homologous recombination using ES cells. for transfection. Correctly targeted ES clones were then used to generate mice heterozygous (*Etv6-CBFA2*$^{+/-}$) and homozygous (*Etv6/CBFA2*$^{-/-}$) for the mutation. Finally, the effect of the *Etv6-CBFA2 fusion* gene on definitive haematopoiesis and embryonic development in both heterozygous and homozygous animals was assessed.

3.1. Identification and Characterisation of Clones Containing Murine Etv6 Exon 5

3.1.1. Isolation of Clones Containing Murine Etv6 Exon 5

The exon organisation of the human *ETV6* and *CBFA2* genes has been discussed in chapter 1. In the t(12;21) translocation the breakpoint always occurs between exons 5 and 6 of the *ETV6* gene and between either exons 1 and 2 of the *CBFA2* gene.[1; 102] Therefore the experimental plan was to isolate a mouse genomic clone containing *Etv6* exon 5 and to insert a cDNA containing exons 2-8 of the human *CBFA2* gene immediately 3' of this exon.

In preparatory work for this project, Dr Foroni had screened a murine embryonic stem cell library (prepared in the λ2001 vector[131; 303]) using a cDNA probe covering the whole of the *ETV6* coding region. Several clones were identified and taken through three rounds of purification. An 18 kb λ phage clone (λES15) containing *Etv6* exon 5 was subsequently isolated by sequencing the coding region of each clone. A restriction map of this mouse genomic clone is represented in Figure 3.1. A 7.5 kb Eco RI fragment was subcloned from λES15 into pBluescript KS^{+}. This 7.5 kb Eco RI (ES15R) fragment was found to correspond to a genomic mouse 7.5 kb Eco RI fragment by comparing the restriction map of the newly identified clone and mouse ES genomic DNA. A probe

spanning the Xho I-Sac I part of the original cDNA probe (bp 305 to 542 of the coding *Etv6* region, GenBank Accession Number Y 07915) was used for this analysis (probe XS 5ETV6).

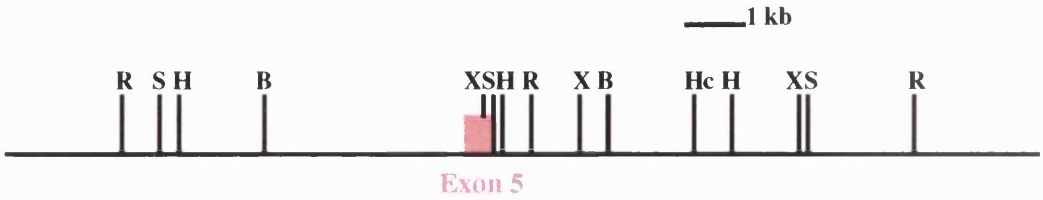


Figure 3.1. Partial Restriction Map of λES15.

The relevant restriction sites shown are: R, Eco RI; S, Sac I; H, Hind III; B, Bam HI; X, Xho I; Hc, Hinc II.

Restriction mapping of the ES15R subclone revealed minimal flanking DNA sequence 3' to exon 5 (Figure 3.1). To maximise the frequency of homologous recombination events in ES cells, the 5' and 3' DNA flanking sequences should be of a similar length and the ES15R subclone was therefore discarded. A 6.0 kb Bam HI genomic subclone, ES15Bam, was then subcloned into pBluescript KS^+ from λES15. Restriction mapping showed that this subclone contained ~3.5 kb genomic DNA sequence 5' of exon 5 and ~2.0 kb 3' to the coding region (Figure 3.2). Comparison of restriction maps between the Bam HI subclone and mouse genomic DNA showed that the Bam HI clone was a suitable and reliable clone for subsequent manipulations. Unique Sac I, Hind III and Eco RI sites were identified for subsequent cloning steps. Digestion of the ES15Bam subclone and filter hybridisation with the probe XS 5ETV6 revealed the presence of a Xho I site within the intronic region of the clone, in addition to the Xho I site within exon 5, the presence of which was confirmed by subsequent sequence analysis (see below). This site would also be used in subsequent cloning steps.

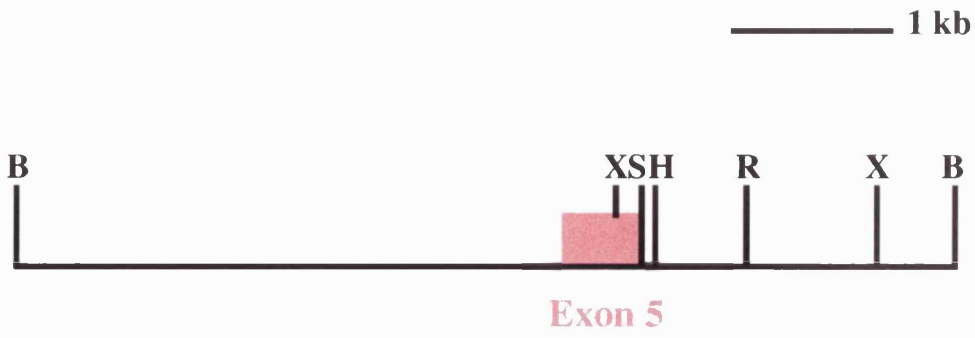


Figure 3.2. Restriction Map of the 6 kb ES15Bam Subclone.

The relevant restriction sites shown are: B, Bam HI; X, Xho I; S, Sac I; H, Hind III; R, Eco RI.

3.1.2. Sequencing Murine Etv6 Exon 5

The full sequence of the murine *Etv6* exon 5 was obtained as follows.

The 6kb ES15Bam pBluescript subclone was sonicated and shotgunned into M13mp18 vector prior to manual sequencing. Clones were selected for sequencing by hybridising filters to the probe XS 5ETV6. The resulting sequence was assembled using a "contig- analysis software" (DNASTAR) to generated a single contiguous overlapping sequence. At this time the sequence of the murine *Etv6* gene (GenBank Accession Number Y 07915) was published. The generated sequence around exon 5 of the ES15Bam clone was compared to the published murine *Etv6* sequence and also to human *ETV6* cDNA sequence (GenBank Accession number U 11732)[92] (Figure 3.3). The internal Xho I site and 3' Sac I site provisionally identified by restriction mapping were located. There was a high degree of homology between the human *ETV6* sequence and the sequence generated from the ES15Bam clone. However, the internal Xho I was not present in the human sequence. The sequence generated from the ES15Bam clone matched the published *Etv6* exon 5 sequence except for a single base (at position 33 in the sequence of exon 5). This was probably a polymorphism (which I did not investigste further) and the change did not result in a change in amino acid.

Figure 3.3. Sequence Analysis of the Murine ES15Bam Clone and Comparison with the Published Human and Murine Sequences of ETV6 Exon 5.

The 3' non-coding sequence is in lower case. The internal Xho I, external Sac I sites and the single base polymorphism are highlighted.

3.2. Generation of the ETV6-CBFA2 Targeting Construct

Once the 6.0 kb ES15Bam subclone was found to be suitable for the purpose of our experiments, a cloning strategy, making use of the identified restriction sites, was devised to generate a targeting construct suitable to be inserted into ES cells by homologous recombination.

3.2.1. Removal of the 3' Xho I Site

It was decided that the Xho I site in exon 5 was suitable for the insertion of the *CBFA2* sequences (i.e. exons 2-8). Therefore, it was necessary to remove the Xho I site lying 3' of exon 5 (Figure 3.2) using site directed mutagenesis. To optimise this step, sequences surrounding the Xho I site were required to design oligonucleotide primers for the site directed mutagenesis.

The 6kb ES15Bam subclone was once again sonicated and shotgunned into M13mp18 vector prior to manual sequencing. Clones were selected by hybridisation to a 1.4 kb intronic probe (ES15Bam3'int) corresponding to the Eco RI and Bam HI fragment 3' of exon 5 (Figure 3.1). Full sequence was generated and forward and reverse oligonucleotide primers were designed to remove the Xho I site and replace it with Cla I (Figure 3.4) by site directed mutagenesis.

Figure 3.4. Sequence of the Area Surrounding the 3' Xho I Site and Oligonucleotide Primers Designed to Replace the Xho I site with Cla I.

Restriction sites are shown in red. The bases to be mutated are underlined.

Since the total size of the plasmid was 9kb (2.97 kb pBluescript and 6kb ES15Bam insert) an extension time of 18 minutes was used. As only 2 bp were mutated 12 thermo-cycles were used. Clones were digested with Xho I and Cla I and one clone was selected in which a Cla I site had replaced the 3' Xho I site (ES15Bam/X$^-$). This subclone was then used for subsequent cloning steps.

3.2.2. Insertion of the Poly-Adenylation Addition Signal

All genes, in order to be correctly processed prior to translation require the presence of a poly-adenylation (poly(A)) recognition sequence following the stop codon. As the *CBFA2* gene was going to be cloned immediately 3' of the *Etv6* exon 5, a poly(A) addition signal needed to be introduced into the ES15Bam/X$^-$ construct downstream from the *CBFA2* coding region in order to terminate transcription of the fusion gene and allow correct post-transcriptional processing. The unique Sac I site situated 11 bp 3' of exon 5 was identified for this use.

The poly(A) recognition sequence was obtained from Promega as part of the pCI vector system (GenBank Accession Number U47119) which contains a 1.2 kb SV40 late poly(A) cassette (Figure 3.5). The poly(A) cassette was extracted by digestion with Eco RI and Bam HI. However, several restriction sites in the multi-cloning site would remain after digestion (Figure 3.5). The pCI vector was therefore initially digested with Xba I and Not I, filled in and re-ligated to remove these unwanted restriction sites.

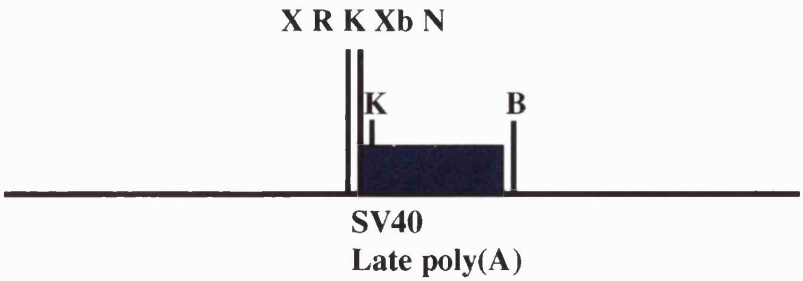


Figure 3.5. Linear Map of the Relevant Region of the 4006 bp pCI Vector.

The SV40 late poly(A) cassette is shown. Relevant restriction enzyme sites shown are: X, Xho I; R, Eco RI; K, Kpn I; Xb, Xba I; N, Not I; B, Bam HI.

In order to use the internal Sac I site, 3' of exon 5, the 6 kb ES15Bam/X$^-$ construct was then subcloned into a Sac I negative pBluescript vector. The 280 bp Eco RI/Bam HI poly(A) fragment from the pCI vector was then blunt cloned into the Sac I site in the ES15Bam/X$^-$ construct. Positive colonies were identified by hybridising filters to the same 280 bp Eco RI/Bam HI poly(A) fragment. These clones were digested with Kpn I/Hind III restriction enzymes to confirm correct orientation. Clones were then digested with Bam HI, Eco RI and Sac I to ensure that all three restriction sites had been successfully removed as a result of the blunt cloning of the poly(A) cassette. Unfortunately, only the Sac I site had been removed with both the Eco RI and Bam HI sites still present in the ES15Bam/X$^-$poly(A) construct. When the ends of the Eco RI/Bam HI digested pCI vector had been filled in prior to blunt cloning the enzyme T4 DNA polymerase was used. T4 DNA polymerase exhibits 3'-5' exonuclease and 5'-3' filling in activity. The Eco RI/Bam HI polyA fragment was end filled in by T4 DNA polymerase and cloned into the filled Sac I site (Figure 3.6). This resulted in the removal of the Sac I site while the Eco RI and Bam HI sites remained.

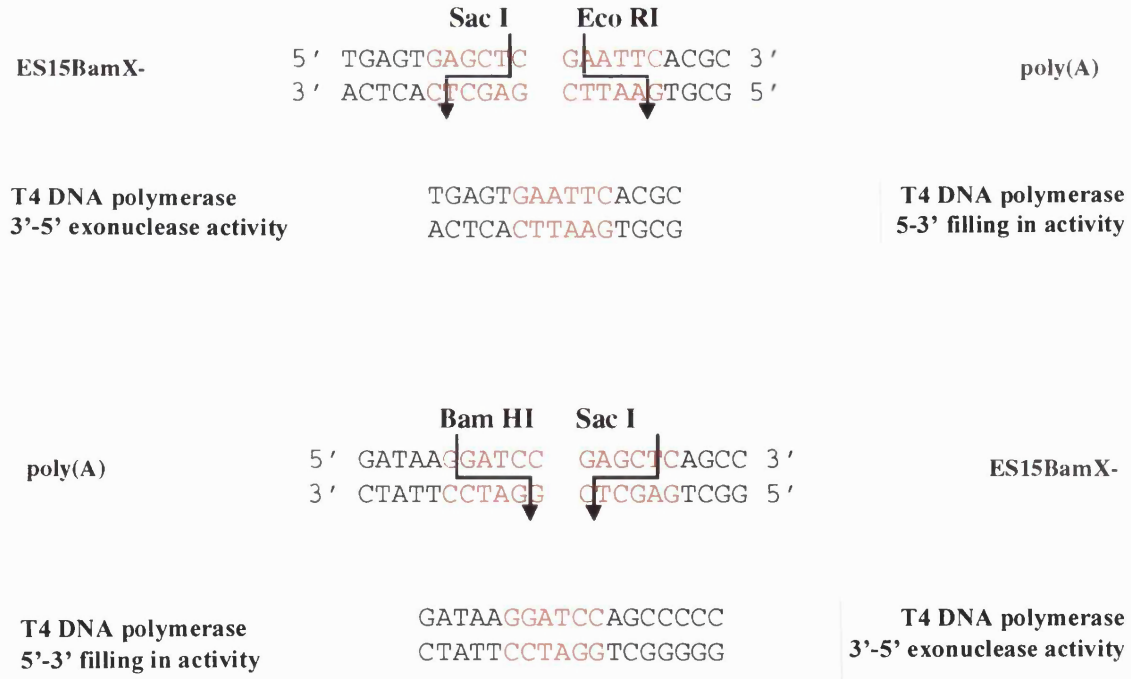


Figure 3.6. Schematic Explanation of the Retention of Bam HI and Eco RI Restriction Sites After Digestion and Filling in with T4 DNA Polymerase.

The retention of the Eco RI site was not important as there were other Eco RI sites within the construct and therefore would not be used for further cloning steps. However, the presence of the Bam HI site was going to interfere with the whole cloning strategy, as Bam HI was the restriction site used for cloning of the original exon 5 fragment, and it had to be removed.

3.2.2.1. Removal of the Bam HI site from the pCI Vector and Reintroduction of the Poly(A) Cassette into the ES15Bam/X⁻ Construct

As stated above it was vital that no Bam HI sites were introduced into the ES15Bam/X⁻ construct. It was decided to remove the Bam HI restriction site by site directed mutagenesis. To achieve this the Bam HI in the pCI vector was first mutated to an Eco RI site with the oligonucleotides shown in Figure 3.7. The total size of the plasmid was 4 kb resulting in an extension time of 12 minutes. As only 2 bp were mutated 12 thermo cycles were used.

```
                    Bam HI
          5' GATAAGGATCCGGGCTGGCGTAATAGCG 3'

Forward   5' GATAAGAATTCGGGCTGGCGTAATAGCG 3'
Reverse   3' CTATTCTTAAGCCCGACCGCATTATCGC 5'
                    Eco RI
```

Figure 3.7. Sequence of pCI Plasmid Surrounding the 3' Bam HI Site.

The forward and reverse primers used for site directed mutagenesis are also shown and the introduced Eco RI site indicated. The 2bp to be mutated are underlined.

Positive colonies were identified by hybridisation to the 280 bp Eco RI/Bam HI poly(A) fragment. These clones were then digested with Eco RI to ensure replacement of the Bam HI site with Eco RI. The subcloning of the 280 bp Eco RI fragment containing

the poly(A) cassette was then repeated into the Sac I site of the ES15Bam/X$^-$ construct, following the blunting of the Eco RI fragment. Positive colonies were again identified by hybridisation to the 280 bp Eco RI/Bam HI poly(A) fragment. These clones were digested with Kpn I/Hind III restriction enzymes to confirm correct orientation. Clones which were correctly integrated were then digested with Bam HI, Eco RI and Sac I to ensure that all Bam HI site had been successfully removed as a result of the blunt cloning of the poly(A) cassette. The two Eco RI sites were retained but both Bam HI and Sac I sites were successfully removed. This construct, ES15Bam/X$^-$poly(A), is shown diagrammatically in Figure 3.8.

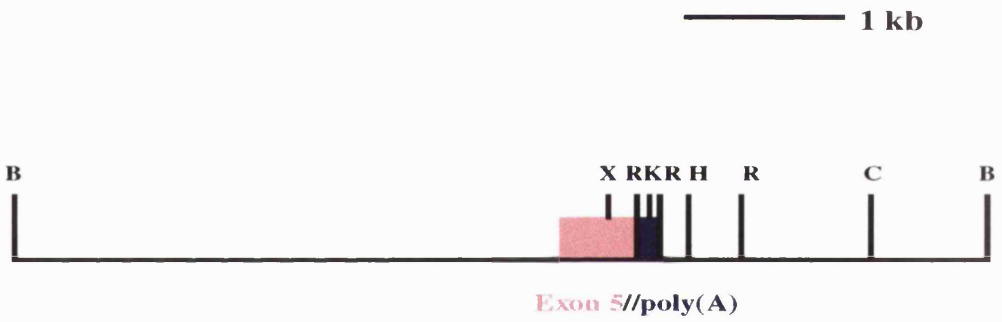


Figure 3.8. Schematic Representation ES15Bam/X$^-$Poly(A) Construct.

Only relevant restriction sites are shown: B, Bam HI; X, Xho I; R, Eco RI; K, Kpn I; H, Hind III; C, Cla I.

3.2.3. Introduction of the Neomycin Resistance Gene

In order to select clones, in which the fusion construct had been successfully placed, it was necessary to introduce a marker that would allow for positive selection. The neomycin resistance gene was used to fulfil this function. Incorporation of the

neomycin resistance gene within the targeting construct confers resistance to the antibiotic G418, in culture. The neomycin (neo) cassette was available in the laboratory as a 1.1 kb Bam HI insert in a pMCI plasmid (Figure 3.9). The cassette had an internal Eco RI site 200 bp downstream from the 5' Bam HI site that would be used to check orientation. It was decided to blunt clone the 1.1kb Bam HI neo cassette into the Hind III site just 3' of the inserted poly(A) cassette. As the neo cassette has its own promoter region and poly(A) addition sequence it was decided to clone it in the opposite orientation to the rest of the insert. This was to avoid any possibility of the neo promoter interfering with transcription of the fusion gene.

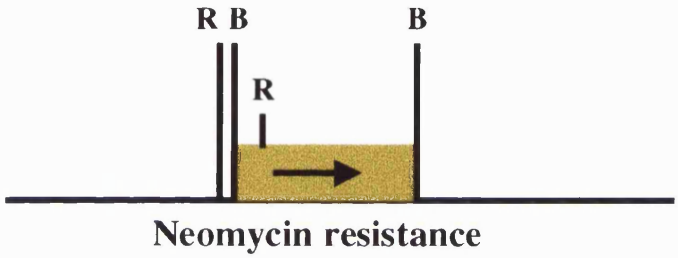


Figure 3.9. Linear Map of the Relevant Region of the pMCI Neo Vector.

The 1.1 kb Neomycin cassette is shown along with the direction of transcription. Relevant restriction enzyme sites are: R, Eco RI; B, Bam HI.

The ES15bam/X$^-$poly(A) clone was initially subcloned into a Hind III negative pBluescript vector. The 1.1 kb neo cassette was then removed from the pMC1 plasmid by digestion with Bam HI. This fragment was then blunt cloned into the unique Hind III site within the ES15Bam/X$^-$poly(A) construct. Positive colonies were selected by hybridising filters to a 0.9 kb neomycin (neo) probe generated by digesting the pMCI neomycin plasmid with Bam HI and Eco RI. Positive clones were digested with Eco RI to check for successful insertion and correct orientation of the neo cassette. This clone was then digested with Bam HI and Hind III to ensure that blunt cloning had removed these sites. This construct, ES15Bam/X$^-$poly(A)neo, is shown diagrammatically in Figure 3.10.

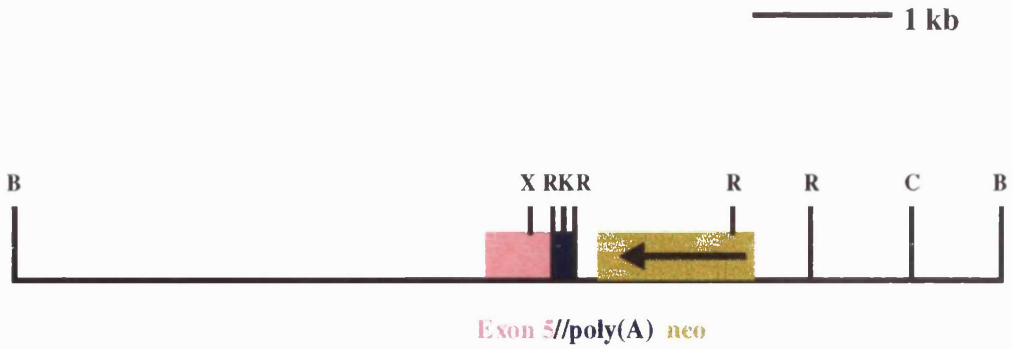


Figure 3.10. Schematic Representation of the ES15Bam/Xpoly(A)neo Cassette.

Etv6 exon 5, the poly(A) and neo cassettes are indicated. The single arrow indicates the direction of transcription of the neomycin gene. Only relevant restriction sites are shown: B, Bam HI; X, Xho I; K, Kpn I; R, Eco RI; C, Cla I.

3.2.4. Introduction of the Herpes Simplex Virus Thymidine Kinase Gene

To enhance homologous recombination, the herpes simplex virus thymidine kinase gene (HSV-tk) was also inserted. Expression of the HSV-tk gene confers sensitivity to the cytotoxic nucleoside analogue 1-(2-deoxy, 2-fluoro-β-Darabinofuanosil)-5-idouracil (FIAU). Clones in which the targeting cassette has been non-homologously recombined retain the HSV-tk gene and are therefore sensitive to FIAU. Homologous recombination, however, results in loss of the HSV-tk gene and resistance to FIAU.

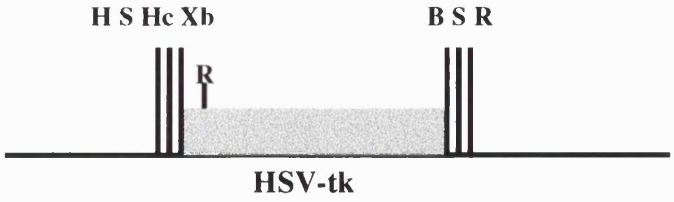


Figure 3.11. Linear Map of the pTK (ES) Construct.

The 2.0 kb HSV-tk cassette is shown. Relevant restriction enzyme sites shown are: H, Hind III; Hc, Hinc II, Xb, Xba I; R, Eco RI; B, Bam HI; S, Sac I.

The HSV-tk cassette was available in the laboratory as part of the pTK (ES) plasmid (Figure 3.11) kindly donated by Dr. TH Rabbitts (Medical Research Council Laboratory of Molecular Biology, Cambridge). This tk cassette was initially removed from the pTK vector by digesting with Hind III and Bam HI. The 2 kb fragment was then cloned into the Hind III/Bam HI sites of a pBluescript vector in which the Xho I site had previously been removed. Positive colonies were selected by hybridising filters to a 1.1 kb probe (tk) generated by digesting the pTK (ES) plasmid with Bam HI and Eco RI. The 7.5 kb ES15bam/X$^-$poly(A)neo cassette was then removed from its original plasmid by Bam HI digestion and cloned into the Bam HI site of the BlueScript-tk plasmid (Figure 3.12). Correct orientation was confirmed by digesting the construct (ES15bam/X$^-$ poly(A)neo-tk) with Eco RI.

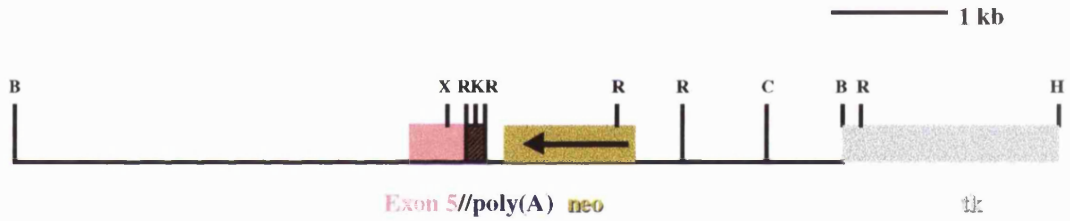


Figure 3.12. Schematic Representation of the ES15bam/X$^-$poly(A)neo cassette in Bluescript-tk Vector.

Etv6 exon 5, the poly(A), neo, and tk cassettes are indicated. The single arrow indicates direction of transcription of the neomycin cassette. Only relevant restriction sites are shown; B, Bam HI; X, Xho I; R, Eco RI; K, Kpn I; C, Cla I; H, Hind III.

3.2.5. Insertion of *CBFA2* cDNA into the ES15Bam/X$^-$poly(A)neo-tk Plasmid

A full-length cDNA clone encoding the *ETV6-CBFA2* fusion product was available in the laboratory. This clone had been isolated from a patient with B-ALL with the t(12; 21) translocation by Dr. O Bernard and had been kindly donated as a 2.5 kb Eco RI fragment (ETV6-CBFA2R) containing the first 5 exons of the *ETV6* gene fused to

exons 2-8 of the *CBFA2* gene. The full sequence of this clone was known and a schematic representation is shown in Figure 3.13.

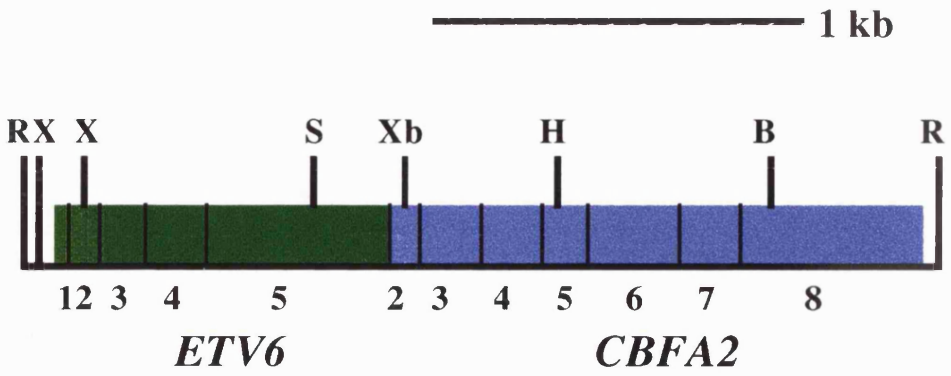


Figure 3.13. Diagrammatic Representation of the 2.5 kb ETV6-CBFA2R Clone.

The numbers represent the individual exons. Restriction sites illustrated are: R, Eco RI; X, Xho I; S, Sac I; Xb, Xba I; H, Hind III; B, Bam HI.

To complete the targeting construct the *CBFA2* cDNA from this ETV6-CBFA2R clone needed to be inserted next to the murine *Etv6* exon 5 in the ES15Bam/X$^-$ poly(A)neo-tk plasmid.

3.2.5.1. Mutation of a Xho I Site into Exon 5 of a Human ETV6-CBFA2 *cDNA Clone*

To mimic the t(12;21) translocation in the targeting construct, we decided to utilise the unique Xho I site within *Etv6* exon 5. An Xho I site would be introduced within the ETV6-CBFA2R clone in exactly the same position as it occurred within the murine exon 5. An Xho I fragment containing the remainder of human *ETV6* exon 5, the *ETV6/CBFA2* fusion, and exons 2-8 of the *CBFA2* gene could then be inserted into the Xho I site in exon 5 of the murine *Etv6* gene. This strategy would guarantee to retain the correct reading frame across the fusion point.

To aid in the mutation of the Xho I site into the ETV6-CBFA2R clone it was decided to sub-clone the 379 bp Sac I/Xba I fragment and insert the new Xho I site by direct mutagenesis.

The 2.5 kb ETV6-CBFA2R clone was initially removed from the plasmid (pcDNAIII, Invitrogen) by Eco RI digestion and ligated into the Eco RI site of a pBluescript plasmid in which the Sac I and Xba I sites had been removed. For future cloning steps the Xho I site in the plasmid poly-linker needed to be 3' of the construct. This was confirmed by Bam HI/ Xho I digestion. The ETV6-CBFA2R clone was digested with Sac I and Xba I. The 379 bp Sac I/Xba I fragment generated was subcloned into another pBluescript plasmid whilst the remainder of the ETV6-CBFA2R clone was also purified and retained. The Xho I site was introduced into the correct position by site-directed mutagenesis by the strategy illustrated in Figure 3.14, which would not produce any amino acid change. The total size of the plasmid was 3.3 kb resulting in an extension time of 7 minutes. As only 2 bp were mutated 12 thermo cycles were used.

Etv6 5' CCCCTGGCAGGAGAGCACTCGAGTGATCCAGCTGATGCC 3'
P W Q E S T R V I Q L M P

ETV6-CBFA2R 5' CCCCCGGCAGGAGAGCACACGCGTGATCCAGCTGATGCC 3'
P R Q E S T R V I Q L M P

Forward primer 5' GGCAGGAGAGCACTCGAGTGATCCAGCTG 3'
Reverse primer 3' CCGTCCTCTCCTGAGCTCACTAGGTCGAC 5'

Figure 3.14. Sequence of Murine Etv6 Surrounding the Internal Xho I Site Compared to the Same Area in the ETV6-CBFA2R Clone.

Single letter translation of the amino acids are shown below the sequence. The bases that were mutated in the ETV6-CBFA2R clone are highlighted. The forward and reverse primers used for site directed mutagenesis are also shown and the introduced Xho I site indicated in red.

The presence of the introduced Xho I site was confirmed by Xho I digestion. The 379 bp Sac I /Xba I fragment containing the introduced Xho I site was then ligated back into the ETV6-CBFA2R clone from which the un-mutated fragment had been removed. Filters were screened using the same 379 bp Sac I/Xba I fragment previously described. The resulting clone, ETV6-CBFA2R/X$^+$, is shown in Figure 3.15.

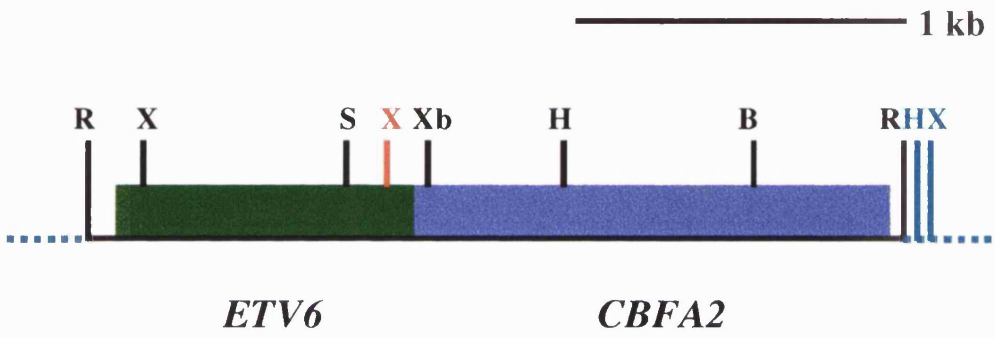


Figure 3.15. Diagrammatic Representation of the ETV6-CBFA2R/X$^+$ Clone.

The introduced Xho I site is shown in red. Plasmid sequence and restriction sites are shown in blue.

3.2.5.2. Insertion of the Human ETV6-CBFA2 Sequence into the ES15Bam/X$^-$ negative Poly(A)neo-tk Plasmid

The ETV6-CBFA2R/X$^+$ clone was digested with Xho I and the 1.6 kb fragment, containing the remainder of *ETV6* exon 5 and exons 2-8 of *CBFA2* (Figure 3.15), was ligated into the Xho I site within *Etv6* exon 5 of the ES15Bam/X$^-$poly(A)neo-tk plasmid. Positive clones were selected by hybridising filters to a 400 bp probe generated by digesting the ETV6-CBFA2R clone with Hind III and Bam HI. Correct orientation of the 1.6 kb Xho I fragment within the final targeting construct [t(12;21) neo] was confirmed by Eco RI digestion. This construct is represented diagrammatically in Figure 3.16.

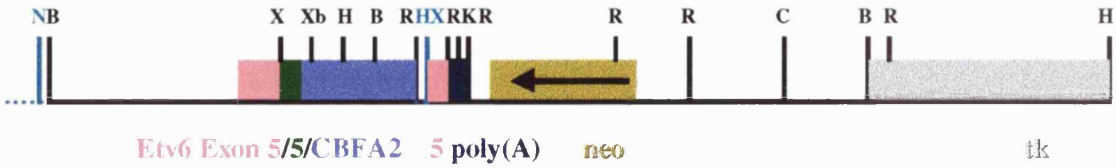


Figure 3.16. Diagrammatic Representation of the t(12;21)neo Targeting Construct.

Exon 5 of Etv6 is shown split where the human sequence is joined to the murine sequence via the introduced Xho I site. Other regions shown are CBFA2 exons 2-8 fused to human ETV6 exon 5, the remainder of murine Etv6 exon 5, and the poly(A), neomycin and tk cassettes. pBluescript sequences and restriction sites are shown in blue. The single arrow indicates the direction of transcription of the neomycin cassette. Not shown to scale. Only relevant restriction sites are shown: N, Not I; B, Bam HI; X, Xho I; Xb, Xba I; H, Hind III; R, Eco RI; K, Kpn I, C, Cla I.

For homologous recombination to occur the targeting DNA needed to be linearised. For this reason the pBluescript Not I site (Figure 3.16) had been conserved during all of the cloning steps previously described. To confirm that this site was unique the t(12;21)neo clone was digested with Not I and a single 14 kb band was identified corresponding to the linearised construct (data not shown).

3.2.6. Sequencing the t(12;21)neo Targeting Construct

Prior to injection into ES cells the t(12;21) targeting construct needed to be sequenced. This was to confirm that: 1) the fusion sequence of murine and human *ETV6* exon 5 and *CBFA2* were in frame and contained no errors; 2) the stop codon was present at the end of exon 8 of *CBFA2*; 3) the poly(A) addition sequence was present; 4) the

neomycin cassette was in place and sited in the opposite direction to the *ETV6/CBFA2* fusion sequence.

To achieve this sequencing primers were designed to cover, in both directions, the whole of the *ETV6/CBFA2* sequence along with the poly(A) and neomycin cassettes. These primers are listed in Appendix II.

The t(12;21) construct was sequenced using an ABI 377 Automated Sequencer (Perkin Elmer). A single contiguous sequence was generated (using DNASTAR software) and compared with the published murine and human *ETV6* and *CBFA2* sequences as well as those of the pMCI Neo and pCI (poly(A)) vectors. Once the t(12;21) construct was found to be free of errors it was prepared for injection into ES cells by linearising 100μg of DNA with Not I. The linearised DNA was then purified with PCIA, precipitated with ethanol, resuspended in sterile saline and quantified by spectrophotometer at OD_{260}nm.

3.2.7. Preparation of Probes for Detection of Homologous Recombination

In order to identify ES cell clones containing homologous recombination events, two unique *Etv6*-specific flanking probes were generated. These genomic sequences lie 5' and 3' to the sequences incorporated in the t(12;21)neo targeting construct, but within a restriction fragment diagnostic of homologous recombination (Figure 3.17).

The 5' external probe was generated by digesting the ES15R clone with Eco RI and Hind III and subcloning the resulting 1 kb fragment into pBluescript. This 5' external probe was hybridised against total mouse genomic DNA digested with Eco RI to ensure that the expected 7.1 kb genomic fragment could be identified and to test for the presence of repetitive sequence elements.

The 3' external probe was generated by digesting λ2001 ES15 DNA with Hind III. These fragments were then shotgunned into pBluescript and the resulting colonies screened by hybridising to the 1.4 kb ES15Bam3'int probe to identify the 3' Hind III clone. This 4.1 kb clone was then digested with Bam HI and Hinc II and the resulting 1.8 kb fragment subcloned into pBluescript. This 3' external probe was hybridised against total mouse genomic DNA digested with Hind III to ensure that the expected 4.1 kb genomic fragment could be identified and to test for the presence of repetitive sequence elements.

Both the 5' and 3' external probes were found to identify the expected bands on hybridisation to mouse total genomic DNA. The probes appeared to be free of repetitive sequences and identified discrete bands of the expected size.

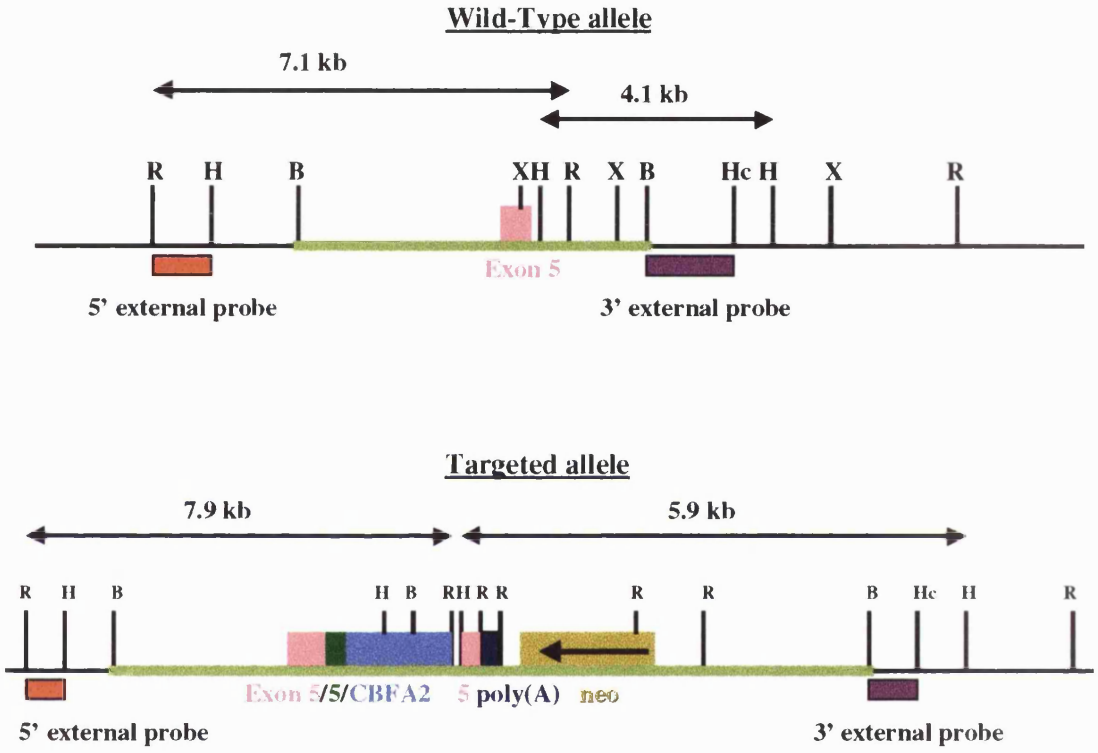


Figure 3.17. Restriction Map of the Etv6 Gene in the Proximity of Exon 5 and the Predicted Structure of the Targeted Etv6 Locus Following a Homologous Recombination Event.

The region covered by the targeting construct is shown as a green line. The horizontal single arrow indicates the direction of transcription of the neomycin cassette. The position of the Etv6/ETV6/CBFA2 fusion gene, the poly(A) and neomycin cassettes, and the probes used for filter hybridisation are indicated. The expected lengths of the restriction fragments diagnostic for homologous recombination are indicated by double headed arrows. Only the relevant restriction sites are shown: R, Eco RI; H, Hind III; B, Bam HI; Hc, Hinc II.

3.3. Generation of Heterozygous Mutant Etv6/CBFA2 $^{+/-}$ Mice

3.3.1. Insertion of the t(12;21)neo Targeting Construct into ES cells by Homologous Recombination

25μg of t(12;21) DNA was electroporated into $2x10^7$ murine CCB clone ES cells (from 129SvEv strain). The electroporation was carried out by Richard Pannell at Medical Research Council Laboratory of Molecular Biology, in Cambridge, UK. Fourty eight hours after the electroporation, cells were subjected to positive (G418) and negative (FIAU) selection and grown for a further 5-6 days. After this time, individual positive colonies could be clearly seen to grow. The above procedure was repeated on two separate plates with different amount of linearised DNA. At this stage I was able to pick discrete colonies which were regrown for DNA extraction. Genomic DNA was isolated and digested with Eco RI. Filters were then hybridised to the 5' external probe. Table 3.1 summarises the results of these two independent experiments. Homologous recombination frequencies of 0 and 3% of the total number of G418/FIAU double-resistant colonies were obtained in the two experiments.

	Cells Electroporated	μg Vector DNA	$G418^r/FIAU^r$ Colonies Screened	Total with Identifiable Genotype	Total Homologous Recombinants	% Homologous Recombinants
1	$2x10^7$	25.0	84	71	0	0
2	$2x10^7$	25.0	140	101	3	3

Table 3.1. Electroporation of ES cells with Etv6 Targeting Vector.

Data from two independent electroporation experiments are given. These involved the targeting vector t(12;21)neo which had been linearised at the unique Not I site. Homologous recombination events were detected by filter hybridisation analysis of DNA isolated from individual G418/FIAU resistant colonies. Not all colonies yielded sufficient DNA to be analysed. The % of homologous recombinants is calculated from the ratio of the number of correctly targeted clones to the total number of G418/FIAU-resistant colonies with an identifiable genotype.

One representative Southern blot is shown in Figure 3.18A. A 7.1 kb fragment corresponding to the wild type band is detected in 15 of the 19 DNA samples analysed. In addition one of these 15 clones displayed a second, larger, band corresponding to the 7.9 kb fragment as predicted for homologous recombination.

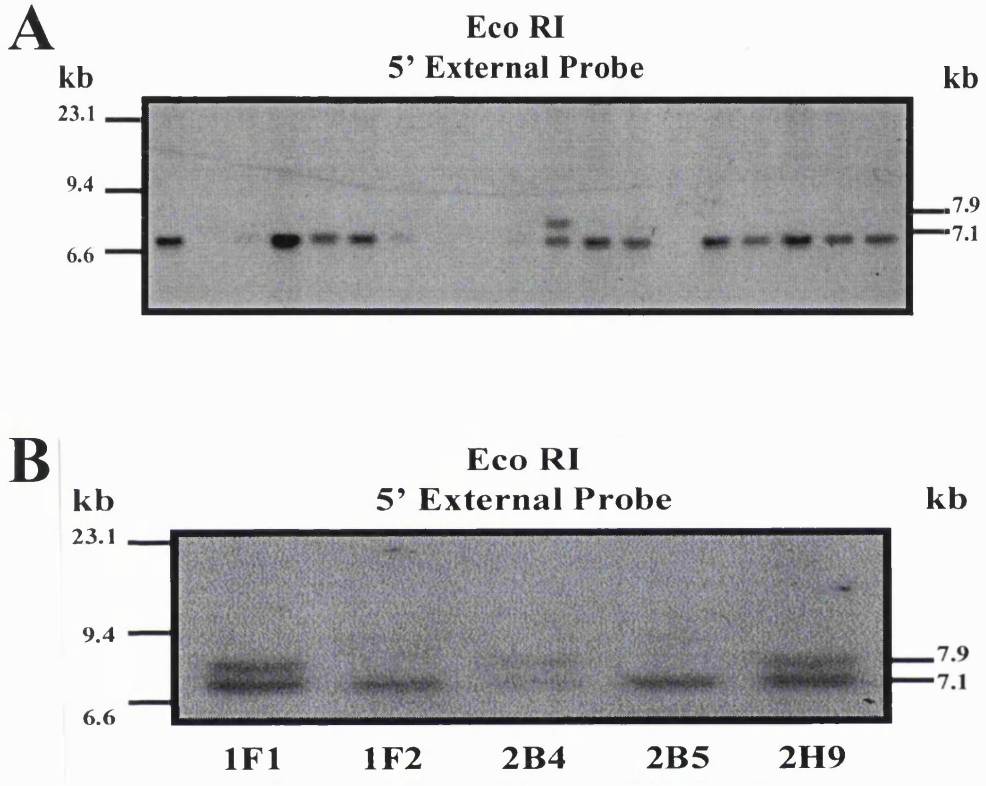


Figure 3.18. Screening for Etv6 Targeted ES Cell Clones.

(A) Representative Southern blot of 20 ES cell clone DNA samples digested with Eco RI and hybridised with the 5' external probe. The 7.1 kb fragment, corresponding to the wild-type allele, is visible in 15 samples and the 7.9 kb fragment, corresponding to the mutant allele, visible in only one.

(B) Confirmation of Targeted Clones.

DNA from the 3 ES clones identified as having a mutated allele along with the clones next to them in the original 96 well plate was digested with Eco RI and re-hybridised to the 5' external probe. The 7.1 kb fragment, corresponding to the wild-type allele, is visible in all 5 samples and the 7.9 kb fragment, corresponding to the mutant allele, is present in 3 (1F1, 2B4, and 2H9).

All three ES cell clones exhibiting the 7.9 kb homologous recombination band were further expanded along with the clones next to them on the original 96 well plate. Genomic DNA was isolated from these clones and again filter hybridised to the 5' external probe (Figure 3.18B) to confirm that the correct clones were selected.

Two independently targeted clones, 1F1 and 2B4, along with one control clone, 1F2, were selected and expanded for further analysis. DNA derived from these clones was digested with Hind III, and filter hybridisation was repeated using the 3' external probe to confirm that these cells indeed contained a mutant *Etv6* allele introduced by homologous recombination. ES cell clones 1F1 and 2B4 exhibited the predicted 5.9 kb mutant and 4.1 kb wild-type Hind III fragments. The control clone, 1F2, displayed a single wild-type fragment (Figure 3.19).

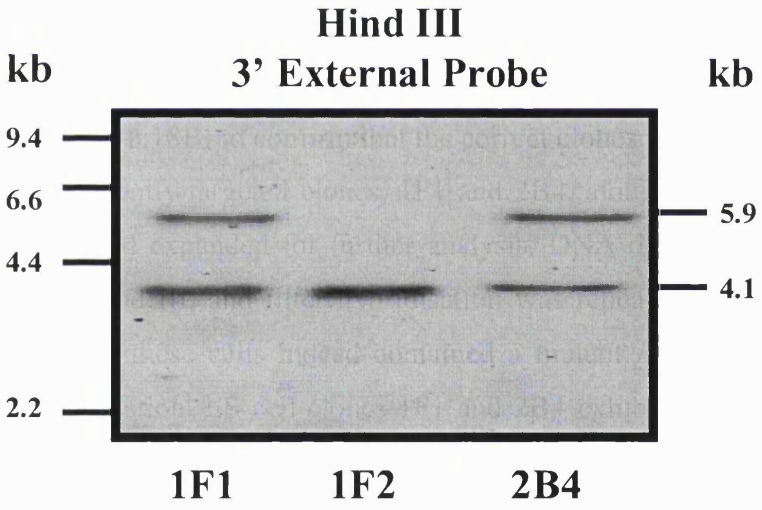


Figure 3.19. Confirmation of Targeted Clones.

DNA from the two selected ES clones (1F1 and 2B4) identified as having a mutated allele along with one wild-type clone (1F2) was digested with Hind III and hybridised to the 3' external probe. The 4.1 kb fragment, corresponding to the wild-type allele, is visible in all 3 samples and the 5.9 kb fragment, corresponding to the mutant allele, is identified in clones 1F1 and 2B4, confirming the presence of the mutant allele in these two clones.

3.3.2. Identification of the Etv6-CBFA2 Fusion Transcript

Having identified two ES cell clones in which the t(12;21)neo construct had been correctly targeted to the *Etv6* locus it was necessary to confirm that the *Etv6/CBFA2* fusion sequence was being transcribed.

The two independently targeted clones, 1F1 and 2B4, and the control clone, 1F2, were expanded and RNA extracted. RNA was also extracted from two established cell lines, EL4 and REH. The EL4 cell line (ATCC number TIB-39) was established from a mouse T-cell lymphoma. The REH cell line (ATCC number CRL-8286) was established from a patient with non-T, non-B cell ALL and was subsequently found to have t(12;21)(p13;q22) translocation with loss of the second ETV6 allele.[304] cDNA was amplified using the OneStep RT-PCR kit (Qiagen) and primers designed to span the *Etv6-CBFA2* fusion point. The Etv6 forward primer (ETV6 4F) was sited to exon 4, i.e. external to the t(12;21)neo targeting construct and was designed to anneal to both the murine and human *ETV6* sequence. The CBFA2 reverse primer (CBFA2 8R) was sited to exon 8. To confirm expression of the second *Etv6* allele and of *CbfA2,* RT-PCR was performed using primers sited to *ETV6* exons 1 (ETV6 1F) and 8 (ETV6 8R) and *CBFA2* exons 1 (CBFA2 1F) and 3/4 (CBFA2 3/4R). All primers were designed to anneal to both human and murine ETV6 and CBFA2 sequences. To confirm RNA and cDNA integrity an RT-PCR reaction was performed using primers designed to detect the ubiquitously expressed glucose-6-phosphate dehydrogenase (G6PD) gene product (G6PD F and G6PD R). These primers anneal to both murine and human *G6PD* sequences. Primers used for RT-PCR are shown in Figure 3.20A. In all reactions 35 cycles were used at an annealing temperature of; 63°C for ETV6-CBFA2, 64°C for ETV6, CBFA2 and G6PD. All products were visualised on ethidium bromide stained 2% (G6PD) and 0.8% (ETV6-CBFA2, ETV6 and CBFA2) agarose gels. The expected 1610 bp *ETV6-CBFA2* amplification product was observed in the REH cell line and in clones 1F1 and 2B4 but not in the EL4 cell line (Figure 3.20B). The predicted 1298 bp *Etv6* amplification product was observed in the EL4 cell line, clone 1F2 and both targeted clones but not in the REH cell line (Figure 3.21C). The two additional bands present in EL4, 1F1 and 2B4 may be splice variants of the *Etv6* gene. The 372 bp CBFA2 amplification product and the 168 bp G6PD product was identified in all five samples (Figures 3.20D and 3.20E). A control reaction in which RNA was replaced by water was negative in all four experiments.

A

ETV6 4F	5′	GGCGACGTGCTCTATGAACT	3′
CBFA2 8R	3′	AACTGGCGCGGGTCGCTGAAC	5′
ETV6 1F	5′	ATGTCTGAGACTCCTGCTCAG	3′
ETV6 8R	3′	CGGTGTGTTCGGCCACTCCATG	5′
CBFA2 1F	5′	GTCTCCCGGGAGCAGCTTGC	3′
CBFA2 3/4R	3′	CCACCTTGAAAGCGATGGGC	5′
G6PD F	5′	ATTCATCATCATGGGTGCATCG	3′
G6PD R	3′	TGTTTGCGGATGTCAGCCACTGT	5′

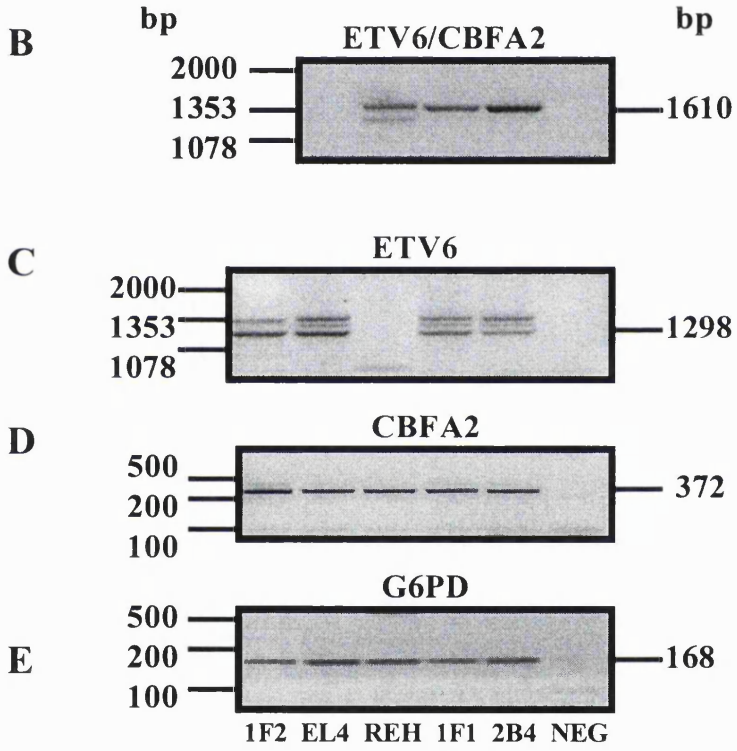


Figure 3.20. Identification of CBFA2, ETV6 and ETV6-CBFA2 fusion products.

(A) Primers used to detect the ETV6-CBFA2 fusion and the ETV6 and CBFA2 products along with the G6PD control product.(B) The REH and ES cell lines 1F1 and 2B4 display the expected 1610 bp ETV6/CBFA2 fusion product, absent in the EL4 cell line. (C) The EL4 and ES cell lines 1F1, 1F2 and 2B4 display the expected 1298 bp Etv6 product. (D) The 372 bp CBFA2 product is amplified in all five samples. (E) The 168 bp G6PD product is amplified in all five samples.

3.3.3. Karyotyping Targeted ES Cell Clones

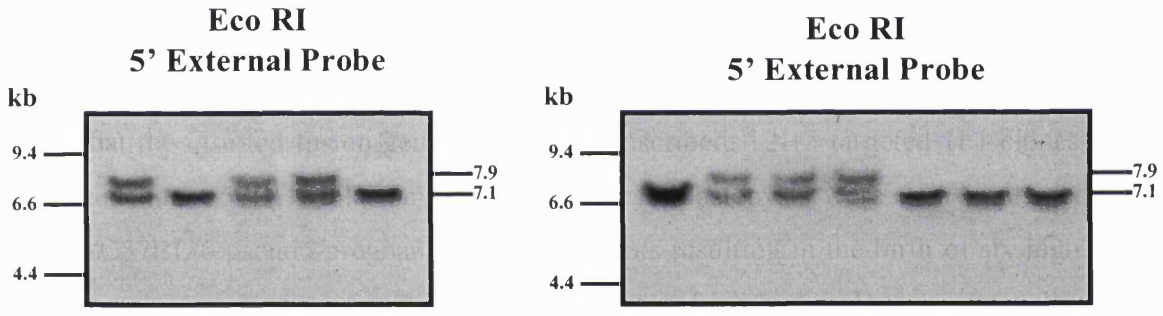
To ensure that the correctly targeted cell clones, 1F1 and 2B4, did not contain any gross chromosomal abnormalities the two clones were karyotyped. This work was carried out by Paul Sinclair in the Department of Haematology, Royal Free Hospital, London. Metaphase spreads prepared from both ES cell clones were shown to have a euploid complement of 40, microscopically normal, chromosomes (Figure 3.21).

Figure 3.21. G-Banded Karyotype of ES Clone 1F1.

40, microscopically normal chromosomes are identified in the metaphase spread.

3.3.4. Generation of Heterozygous and Homozygous Mutant Etv6/CBFA2 Mice

Having demonstrated that the 1F1 and 2B4 ES cell clones were correctly targeted and that the inserted fusion gene was being transcribed, 12-15 targeted 1F1 clones were injected into C57BL/6 blastocysts. 8-12 of these blastocysts were then implanted into CBA/C57BL/6 pseudo-pregnant female recipients resulting in the birth of six high level chimeras (3 male and 3 female). This work was carried out by Richard Pannell, at the MRC LMB laboratory in Cambridge, UK. The high percentage male chimeras were crossed with CBA/C57BL/6 females in order to transmit the *Etv6–CBFA2* mutation through the germline. Germ-line transmission of the *Etv6-CBFA2* mutation was confirmed by filter hybridisation analysis of tail biopsy DNA from 21 day old pups using the 5' external probe on Eco RI digested DNA (Figure 3.22).



Figue 3.22. Confirmation of Germ-Line Transmission of the Etv6-CBFA2 Mutation.

Filter hybridisation of Eco RI digested mouse tail biopsy DNA with the 5' external probe from 21 day old pups, derived from two independent crossings, yields fragments of 7.1 kb (wild-type allele) and 7.9 kb (mutant allele) in six of the twelve samples.

A total of 36 mice generated by crossing chimeric males with CBA/C57BL/6 females have so far been genotyped, resulting in 18 being identified as heterozygous for the *Etv6-CBFA2*. A further 5 of 23 mice generated by crossing heterozygous *Etv6-CBFA2* males with CBA/C57BL/6 females have also been genotyped as heterozygous (Table3.2).

Table 3.2. Details of Mice Generated by the Indicated Matings

Cross	Mouse	DOB	Sex	Genotype	Age (Mo)
	16001	7/5/99	M	Chimera	14.5
	16001	7/5/99	M	Chimera	14.5
	16002	7/5/99	M	Chimera	14.5
	16003	7/5/99	F	Chimera	14.5
	16004	7/5/99	F	Chimera	14.5
	16005	7/5/99	F	Chimera	14.5
16000 X C57	16006	21/9/99	M	+/-	10.0
	16007	21/9/99	M	+/+	10.0
	16008	21/9/99	M	+/-	10.0
	16009	21/9/99	F	+/-	10.0
	16010	21/9/99	F	+/+	10.0
16001 X C57	16011	25/9/99	M	+/+	9.8
	16012	25/9/99	M	+/-	9.8
	16013	25/9/99	M	+/-	9.8
	16014	25/9/99	M	+/-	9.8
	16015	25/9/99	F	+/+	9.8
	16016	25/9/99	F	+/+	9.8
	16017	25/9/99	F	+/+	9.8
16002 X C57	16018	8/10/99	M	+/-	9.4
	16019	8/10/99	M	+/+	9.4
	16020	8/10/99	F	+/+	9.4
	16021	8/10/99	F	+/+	9.4
	16022	8/10/99	F	+/-	9.4
	16023	8/10/99	F	+/-	9.4
	16024	8/10/99	F	+/+	9.4
16000 X C57	16025	14/10/99	M	+/-	9.2
	16026	14/10/99	M	+/+	9.2
	16027	14/10/99	F	+/+	9.2

Table 3.2. (cont. 1).

Cross	Mouse	DOB	Sex	Genotype	Age (Mo)
16000 X C57	16028	14/10/99	F	+/+	9.2
	16029	14/10/99	F	+/+	9.2
	16030	14/10/99	F	+/+	9.2
16002 X C57	16031	30/10/99	M	+/-	8.7
	16032	30/10/99	M	+/+	8.7
	16033	30/10/99	M	+/-	8.7
	16034	30/10/99	M	+/-	8.7
	16035	30/10/99	M	+/-	8.7
	16036	30/10/99	M	+/-	8.7
	16037	30/10/99	M	+/-	8.7
	16038	30/10/99	M	+/+	8.7
	16039	30/10/99	F	+/-	8.7
	16040	30/10/99	F	+/+	8.7
	16041	30/10/99	F	+/-	8.7
16008 X C57	16048	16/12/99	M	+/+	7.1
	16049	16/12/99	M	+/-	7.1
	16050	16/12/99	M	+/+	7.1
	16051	16/12/99	M	+/+	7.1
	16052	16/12/99	M	+/+	7.1
	16053	16/12/99	F	+/+	7.1
	16054	16/12/99	F	+/+	7.1
	16055	16/12/99	F	+/-	7.1
16008 X C57	16058	18/01/00	M	+/-	6.1
	16059	18/01/00	M	+/+	6.1
	16060	18/01/00	F	+/+	6.1
	16061	18/01/00	F	+/-	6.1
	16062	18/01/00	F	+/+	6.1
	16063	18/01/00	F	+/-	6.1
	16064	18/01/00	F	+/+	6.1

Table 3.2. (cont. 2).

Cross	Mouse	DOB	Sex	Genotype	Age (Mo)
16008 X C57	16065	18/01/00	F	+/+	6.1
16008 X C57	16066	10/03/00	M	+/+	4.4
	16067	10/03/00	M	+/+	4.4
	16068	10/03/00	M	+/+	4.4
	16069	10/03/00	F	+/+	4.4
	16070	10/03/00	F	+/+	4.4
	16071	10/03/00	F	+/+	4.4
	16072	10/03/00	F	+/+	4.4
16006 X 16009	16042	15/12/99	M	+/+	7.2
	16043	15/12/99	M	+/-	7.2
	16044	15/12/99	M	+/+	7.2
	16045	15/12/99	M	+/+	7.2
	16046	15/12/99	F	+/-	7.2
	16047	15/12/99	F	+/-	7.2
16006 X 16009	16056	18/01/00	F	+/-	6.1
	16057	18/01/00	F	+/-	6.1
16006 X 16009	16076	31/03/00	M	+/+	3.7
	16077	31/03/00	M	+/+	3.7
	16078	31/03/00	F	+/-	3.7
	16079	31/03/00	F	+/-	3.7
	16080	31/03/00	F	+/+	3.7
16006 X 16009	16081	22/04/00	M	+/-	3.0
	16082	22/04/00	F	+/-	3.0
	16083	22/04/00	F	+/+	3.0
	16084	22/04/00	F	+/+	3.0

At 14.5 and 10 months old respectively, none of the six high level chimeric mice or 23 *Etv6-CBFA2* heterozygous animals have displayed any overt phenotype. To date they are healthy and fertile. Heterozygous animals were therefore interbred and the genotypes of the offspring determined by filter hybridisation analysis of Eco RI digested tail biopsy DNA samples isolated 21 days post-natally. From a total of 17 live-born mice analysed to date, 9 heterozygous and 8 wild-type have been identified but no homozygous mutant offspring have been detected. Average litter size was only 5-6 and consequently the present data are strongly suggestive of the homozygous *Etv6-CBFA2* mutation being lethal during the embryonic stage (Table 3.2).

To further investigate the fate of homozygous *Etv6-CBFA2* offspring, two pregnant females, from *Etv6-CBFA2* heterozygous crosses, were killed at day 10.5 of embryonic life (dE10.5) and day 11.5 (dE11.5). The embryos were separated from the placenta and removed from the embryonic membrane. The embryos were stored in formaldehyde prior to being photographed. DNA was extracted from the embryonic membrane, which is of foetal origin, and the genotype determined by filter hybridisation of Eco RI digested DNA probed with the 5' external probe. A total of 19 embryos were genotyped, with 7 displaying only the 7.1 kb germline band, 6 both the 7.1 kb germline and 7.9 kb mutant bands and 6 displaying only the 7.9 kb mutant band (Table 3.3) (Figure 3.23). Five embryos failed to yield enough good quality/quantity DNA for analysis.

dE10.5			**dE11.5**		
Germline	**Heterozygous**	**Homozygous**	**Germline**	**Heterozygous**	**Homozygous**
3	2	3	4	4	3

Table 3.3. Genotype of Embryos Analysed at dE10.5 and dE11.5.

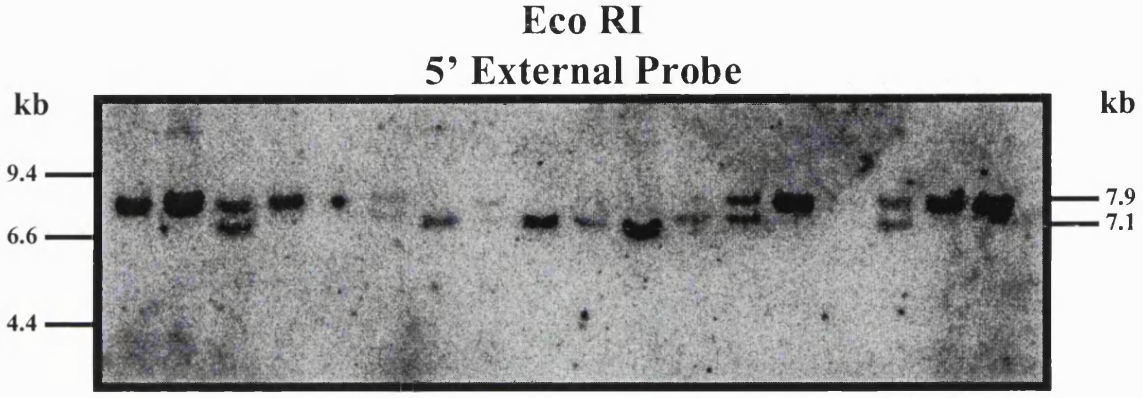


Figure 3.23. Genotyping dE10.5 and dE11.5 Embryos

Representative Southern blot of 16 embryonic sac DNA samples digested with Eco RI and hybridised with the 5' external probe. The 7.1 kb fragment, corresponding to the wild-type allele, is visible in 5 samples. Both fragments (7.1 kb wild type and 7.9 kb mutant) corresponding to the heterozygous genotype are visible in 5 samples and a single 7.9 kb mutant fragment corresponding to the homozygous genotype visible in 6 samples.

Embryos shown genotypically to be homozygous *Etv6-CBFA2* mutants were readily distinguishable phenotypically from the heterozygous or wild-type embryos. They were smaller in size, pale and with no heart or blood flow visible macroscopically or microscopically as illustrated below.

3.3.5. Phenotypic Analysis of Homozygous Mutant Etv6-CBFA2$^{-/-}$ Embryos

Consistent abnormalities were seen in the homozygous *Etv6-CBFA2* embryos analysed at dE10.5 and dE11.5 from litters derived from heterozygous *Etv6-CBFA2* matings. All six embryos identified as being homozygous *Etv6-CBFA2* were extremely runted and very pale (Figure 3.23).

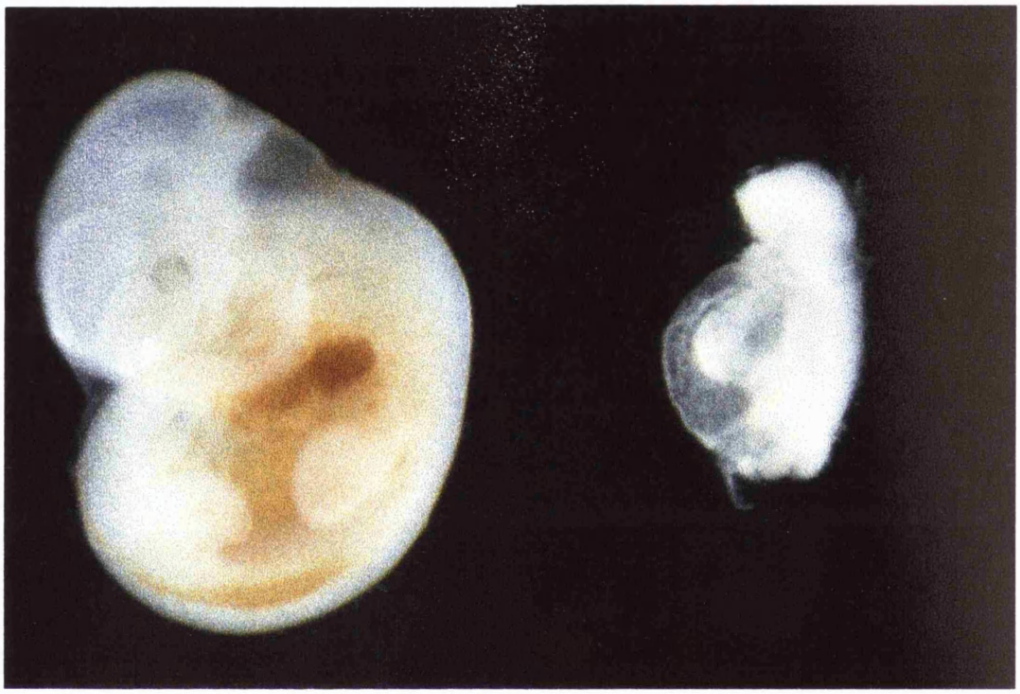


Figure 3.23. Phenotype of Embryos Resulting From Crossing Etv6-CBFA2 Heterozygous Mice.

Embryos from the same litter at dE11.5. The embryo homozygous for the Etv6-CBFA2 mutant allele (Etv6-CBFA2$^{-/-}$) is grossly retarded and very pale compared with the heterozygous (Etv6-CBFA2$^{+/-}$) litter-mate.

These data indicate that the *Etv6-CBFA2*$^{-/-}$ mutation is recessive and embryonic lethal at around dE10.5 of gestation.

DISCUSSION

Chromosomal translocations are the most common genetic abnormalities seen in haematological malignancies. In recent years *in vivo* models have been developed to investigate the role that genetic events associated with translocations play in tumour formation to achieve a more comprehensive understanding of the biology of cancer. Animal models of tumourgenesis offer one means of achieving this. The most frequently used approach is transgenesis.[305] This approach is relatively rapid but efficacy depends on the choice of correct promoter controlling the transgene expression. More recently, new technologies based on homologous recombination in ES cells have been used to mimic chromosomal translocations. Homologous recombination is used to recreate *in vivo,* in the animal the same genetic abnormality observed in human leukaemic cells, retaining the expression of the newly inserted DNA under the control of endogenous sequences, much the same as the fusion gene would be controlled after the specific chromosomal translocation. This approach has been used to investigate the mechanistic role of translocation encoded fusion products associated with a variety of haematological malignancies.[225; 249; 300] Using this approach, chimeric mice containing an in-frame fusion of *AF9* with *Mll* were generated. Mice carrying the fusion gene developed tumours within 12 months at the chimeric stage.[300] The proliferative disorder observed in the animals was similar to that observed in humans, confirming that this strategy is a valuable technique in the study of the pathogenicity of chromosomal translocations.[300] At least two other examples have been recently described using this approach [225; 249] with less impressive results. It is likely that the differences in outcome of these different models depend on the impact of these fusion genes in the leukaemogenic process rather than simple technicalities. The primary or secondary role of these different fusions, as well as their relationship to other events (genetic or chemically induced) could be highlighted by these experiments.

The t(12;21)(p13;q22) translocation, in which the first five exons of the *ETV6* gene are fused to almost the entire *CBFA2* gene, is the most common childhood genetic abnormality.[2] Furthermore, both the *ETV6* and *CBFA2* genes are frequent targets in other chromosomal translocations involved in haematopoietic malignancy.[129] The work described in this thesis involved the investigation of the mechanistic role of the t(12;21)-encoded ETV6-CBFA2 chimeric product in leukaemogenesis. To achieve this, we used

gene targeting to create mice with an *Etv6-CBFA2* knock-in allele that mimics the t(12;21). An artificial fusion construct containing the human *ETV6-CBFA2* fusion gene was generated. Gene targeting in ES cells was then used to introduce this fusion gene into the mouse *Etv6* gene locus. Targeted ES cell clones were shown to contain the *Etv6-CBFA2* fusion gene sited to the *Etv6* locus. Furthermore, the mutant *Etv6-CBFA2* fusion gene was shown to be transcribed from endogenous *Etv6* control elements in two of these clones.

4.1. The *Etv6-CBFA2* Fusion Gene Does Not Cause Leukaemia in Chimeric Mice

Chimeric mice, generated by microinjection of targeted ES cells into blastocysts, remained well 14.5 months after birth. This lack of phenotype in *Etv6-CBFA2* chimeric animals is in marked contrast to the *MLL-AF9* knock-in model, which led to the development of leukaemia by 12 months in 90% of chimeric mice.[300] However, chimeric mice containing the fusion oncogene *CBFβ-MYH11*, generated by a chromosome 16 inversion in human AML M4Eo, were also leukaemia free,[249] as were chimeric mice containing the *CBFA2-MTG8* fusion gene generated by t(8;21) and seen in up to 15% of AML M2.[225] In both latter models, germ-line transmission of the fusion oncogene resulted in embryonic death at around dE12.5-dE13.5 of animals heterozygous for the mutation. These animals died from a complete absence of normal foetal liver-derived definitive haematopoiesis and from haemorrhages.[225; 249] This phenotype was similar to that seen following homologous disruption of either *Cbfa2* or *Cbfβ*.[194; 195; 209; 226] One possibility for the absence of leukaemia in both *Cbfβ-MYH11* and *Cbfa2-MTG8* chimeric mice might be the severe defects of embryonic haematopoiesis resulting in an absence of fusion gene involvement in chimeric haematopoiesis. In both examples the fusion oncogene was targeted to the CBF locus resulting in disruption of normal CBF function. However, the *Etv6-CBFA2* fusion oncogene was targeted to the *Etv6* locus resulting in loss of normal *Etv6* function but retention of both *Cbfa2* alleles. Although embryos heterozygous for the *Etv6* knock-out have been shown to be phenotypically normal,[260] it could not be discounted that the absence of leukaemia in *Etv6-CBFA2* chimeric mice was due to defects in embryonic development and a subsequent lack of involvement in chimeric haematopoiesis.

4.2. Heterozygous Expression of the *Etv6-CBFA2* Fusion Gene Does Not Disrupt Normal Development or Give Rise to Leukaemia

Mice heterozygous for the *Etv6-CBFA2* knock-in allele displayed no overt phenotype and remained well 10 months after birth. This phenotype was similar to that seen in heterozygous *Etv6* knock-out mice,[260] and contrasted with the phenotype observed in the *Cbfβ-MYH11* and *Cbfa2-MTG8* knock-in models.[225; 249] These data suggested that heterozygous expression of the *Cbfa2-ETV6* fusion gene does not affect embryonic development in a dominant fashion. Furthermore, the lack of phenotype in these mice implies that the *Etv6-CBFA2* fusion is not oncogenic when present as a single genetic abnormality.

4.3. Homozygous Expression of Etv6-CBFA2 is Embryonic Lethal

When mice heterozygous for the *Etv6-CBFA2* fusion gene were crossed, no mice carrying the homozygous *Etv6-CBFA2* mutation were identified. When embryos were examined at 10.5 and 11.5 days of gestation, some foetuses appeared underdeveloped and pale. Genotyping showed that all of these animals were homozygous for the *Etv6-CBFA2* mutation, whilst all of the normally developed embryos were wild-type or heterozygous for the *Etv6-CBFA2* mutation. These data indicate that homozygous *Etv6-CBFA2* mice are embryonic lethal and die between dE10.5-dE11.5. This phenotype is strikingly similar to that associated with homozygous disruption of the *Etv6* gene.[260] In those experiments, *Etv6* exons 5, 6 and a portion of exon 7, containing the DNA-binding domain, were replaced with a PGK-neo cassette. As previously mentioned, heterozygous $Etv6^{+/-}$ mice displayed no overt phenotype. However, *Etv6* knock-out mice ($Etv6^{-/-}$) died between dE10.5-dE11.5 with defective yolk sac angiogenesis and intra-embryonic apoptosis of mesenchymal and neural cells. These mice appeared grossly retarded and very pale.[260] In the *Etv6-CBFA2* knock-in model, exons 6–8 of the *Etv6* gene (containing the DNA-binding domain) were replaced with *CBFA2* sequences, resulting in loss of *Etv6* specific function. Taken together, these data suggest that a homozygous disruption of the DNA binding domain of *Etv6* leads to lack of transcription of *Etv6* dependent genes involved in normal development and specifically in those involved in maintaining blood vessel integrity within the developing yolk sac and for survival of different cell types in the developing embryo.[260]

4.4. Heterozygousous Expression of Etv6-CBFA2 is Insufficient for Leukaemogenesis

The absence of any overt phenotype in *Etv6-CBFA2* chimeras or heterozygous knock-in mice suggests that t(12;21) by itself is not oncogenic and that other secondary events contribute to the progression of *ETV6-CBFA2* associated leukaemia. The recent finding that haploinsufficiency of CBFA2 is responsible for an autosomal dominant congenital platelet defect and predisposes to the development of leukaemia[198] suggests that loss of function of CBFA2 may be one such secondary event. In patients with t(12;21) associated ALL, one *CBFA2* allele is disrupted by the reciprocal translocation, whereas the knock-in mouse model described in this work has both *Cbfa2* loci intact. Discounting this hypothesis are the data derived from identical twins, each containing an identical *ETV6-CBFA2* fusion. Although the *ETV6-CBFA2* fusion was shown to be generated in utero, one twin in each pair developed ALL at a much earlier age than the second twin.[274; 275] As both twins contained an identical genetic abnormality, the long latency period between the generation of the fusion and the development of leukaemia suggests that secondary events other than disruption of one *CBFA2* allele are required for the development of the leukaemic phenotype.

The non-translocated *ETV6* allele is frequently deleted in cases of ALL with t(12;21).[265-267] and loss of heterozygosity at the *ETV6* locus is common in childhood ALL.[268-270] These results indicate that disruption of the second *ETV6* allele may be the required secondary event leading to a leukaemic phenotype in association with t(12;21). Techniques are now available to induce secondary genetic events in mouse models. *Cbfβ-MYH11* chimeric mice do not develop tumours in their first year of life.[249] However, when 4-16-week old *Cbfβ-MYH11* chimeric mice were injected with N-ethyl-N-nitrosourea (ENU), a potent DNA alkylating mutagen, 84% of the treated chimeras developed leukaemia 2-6 months after treatment, whereas none of the ENU-treated control animals, lacking the knock-in fusion gene, developed leukaemia.[250] A similar approach could be used to see if artificially induced secondary genetic events lead to leukaemia in the *Etv6-CBFA2* knock-in mouse. Any treatment related tumours could be investigated for the presence of the *Etv6-CBFA2* fusion gene and any abnormalities in the second *Etv6* allele. Although these experiments would answer the question as to

whether secondary genetic events are required for leukaemic progression, the nature of the mutations created are likely to be more random and not truly mimic the mutations involved in patents with t(12;21).

The initial genetic event in t(12;21) associated B-cell ALL is thought to be the fusion of the *ETV6* gene to *CBFA2*. This translocation occurs early in life and is often detected in utero.[274; 275] Because of the sometimes extended latency between identification of the ETV6-CBFA2 fusion and the onset of leukaemia, the second genetic event probably occurs some time after the initial translocation. Also, because t(12;21) is only seen in immature B-cell ALL, these two events must, in some way, preferentially target B-cell precursors. Techniques are now available which allow for the selective modification of genes in a tissue and stage specific manner. The Cre/*loxP* recombination system of bacteriophage P1[306; 307] has been adapted to function in ES cells. In the presence of Cre, any DNA flanked by *loxP* sites is either deleted or inverted, depending upon the orientation of the *loxP* sites. Using this technology, it would be possible to generate a mouse model in which the *Etv6-CBFA2* fusion gene is expressed in B-cell precursors in which the second *Etv6* allele had been disrupted. To achieve this, three separate mouse strains would be required. First, homozygous mice would be generated in which *Etv6* sequences were flanked by *loxP* sites in such a way that removal of the intervening sequence would result in loss of *Etv6* function. These mice would be fully viable as the *Etv6* alleles would only be disrupted in the presence of Cre recombinase. A second strain of mice would be generated in which the *Cre* gene is expressed under the control of a B-cell specific promoter. *CD19* is a suitable candidate since it is only transcribed in cells of B lineage.[308] Moreover, *CD19* is expressed at the earliest stages and throughout B cell development and differentiation.[309] Heterozygous *CD19-Cre* mice would be crossed with the heterozygous *Etv6-CBFA2* line of mice described in this thesis. One in eight of the offspring would contain *CD19-Cre* and will be heterozygous for *Etv6-CBFA2*. These mice would be crossed with homozygous *Etv6-loxP* mice. One in four of the offspring would contain one *Etv6-loxP* and one *Etv6-CBFA2* allele and also contain *CD19-Cre*. The hypothesis is that these animals would be viable as the second *Etv6* allele would only be disrupted in developing B cells, allowing for normal embryonic development. These B cells would then contain the *Etv6-CBFA2* fusion gene and loss of function of the second Etv6 gene thus mimicking the genotype identified in childhood t(12;21) B-ALL. The feasibility of this approach has recently been confirmed when a *Cre*

expression cassette was inserted into the second exon of *CD19* by homologous recombination in ES cells.[310] Mice heterozygous for the *Cre* insertion retained one functional *Cd19* allele and were phenotypically normal. In a model system involving the cross of *CD19-cre* heterozygous mice with mice bearing a *loxP*-flanked substrate, a deletion efficiency of 75-80% in bone marrow-derived pre-B cells increasing to 90-95% in splenic B cells was achieved.[310]

CONCLUSIONS

In this thesis we have demonstrated that the *Etv6-CBFA2* fusion gene resulting from the t(12;21) translocation identified in childhood ALL is not, by itself, oncogenic. Furthermore, homozygous disruption of the *Etv6* DNA binding domain resulted in embryonic lethality, which was phenotypically very similar to that seen in *Etv6* negative animals. However, the *Etv6-CBFA2* mice generated in this project will be used in future projects designed to further elucidate the role of the t(12;21) translocation in leukaemia.

APPENDIX I

Materials (listed in alphabetical order)

All chemicals were puchased from Sigma-Aldrich, Poole, Dorset, UK unless otherwise stated.

AGB Buffer (50x)

242g Trisma base

57.1ml glacial acetic acid

100ml 0.5M EDTA pH 8.0

to 1 litre with DDW

Automated Sequencing Buffer

200mM Tris. PH 7.5

100mM magnesium chloride

250mM sodium chloride

"Carlo's" 10x Restriction Buffer

330µl 1M Tris-acetate pH 7.5

660µl 2M K acetate pH 7.5

10µl Mg acetate

1 mg/ml BSA fraction V

Church Filter Hybridisation Buffer

500ml 1 M Na_2HPO_4 pH 7.2

10g BSA

70g SDS

2 ml 0.5M EDTA pH 8.0

to 1 litre with DDW

DNA Gel Loading Buffer (6x)

50% glycerol

0.2% bromophenol blue

0.2% xylene cyanol

0.1M EDTA pH 8.0

DnD

1M DTT

90% DMSO

10mM Potassium Acetate

1M Dithiothreitol (DTT)

3.09g DTT in 20ml 0.01M Na acetate (pH 5.2)

filter sterilise

Dilute with DDW for 0.1M DTT used in all reactions

Gel Denaturing Solution

1.5M NaCl

0.5M NaOH.

Gel Depurination Solution

20ml of HCl in 1 litre DDW

Gel Neutralising Solution

1.5M NaCl

0.5M Trisma base pH7.2

20% Glucose

20g glucose in 100ml of DDW

sterilised by filtration

GTE Buffer

50mM glucose

25mM Tris. pH 8.0

1mM EDTA pH 8.0

Lambda Dilution Buffer (λ dil)

10mM Tris. pH 7.5

10mM $MgSO_4$

filter sterilised

Leishman Stock Solution

2g Leishman's stain in 1l methanol

dissolve at 70°C for 24 hours with constant mixing and filtered prior to use

L-Broth (LB) (Luria-Bertani)

10g bactotryptone

5g bacto-yeast extract

10g NaCl

to 1 litre with DDW

LB Plates

1.5% bacto-agar in LB

sterilise by autoclaving

solid media melted by microwaving and cooled to 50°C prior to addition of antibiotics

1M $MgSO_4$

24.6g $MgSO_4.7H_2O$ in 100ml DDW

sterilise by autoclaving

20% Maltose

20g maltose in 100ml DDW

sterilise by filtration

2M NaOH

8g NaOH in 100ml of DDW

sterilise by autoclaving

Phenol/chloform/isoamyl alcohol (PCIA)

25ml phenol pH 8.0

24ml chloroform

1ml isoamyl alcohol

20% PEG 2.5M NaCl

40g PEG

29.22g NaCl

dissolve in 100ml DDW, filter and make up to 200 ml final volume with DDW

Salmon Sperm DNA

5mg/ml salmon sperm DNA in ddw

add 10.5mM of NaOH

boil for 10 minutes

(20%) SDS

20g of lauryl sodium dodecyl sulphate in 100 ml DD

Sequence Reaction Buffer (5x)

200mM Tris. pH 7.5

100mM $MgCl_2$

250mM NaCl

Sequence Reaction Buffer (2.5x)

200mM Tris. PH 7.5

5mM $MgCl_2$

pH 9.0 at room temperature

SOB

20g bacto-tryptone

5g bacto-yeast extract

0.5g NaCl

10ml 250mM KCL

adjust to pH 7.0

to 1 litre with DDW

SSC (20x)

3M NaCl

0.3M sodium citrate

adjust to pH 7.0 with NaOH

Stop Solution

95% formamide

20mM EDTA

0.05% bromophenol blue

0.05% xylene cyanol

TBE (5x)

27g Trisma base

13.75g boric acid

10ml 0.5M EDTA pH 8.3

to 500 ml with DDW

TBE Acrylamide Solution (0.5x)

75ml 40% acrylamide/bis-acrylamide

50ml 5x TBE

230g urea

to 500ml with DDW

TBE Acrylamide Solution (2.5x)

37.5 ml 40% acrylamide/bis-acrylamide

125ml 5x TBE

115g Urea

to 250ml DDW

TE Buffer

10mM Tris. pH 8.0

1mM EDTA pH 8.0

TFB

0.5M MES (2-(N-morpholino)ethanesulphonic acid)

to pH 6.3 with KOH

100mM KCl

45mM $MnCl_2$

10mM $CaCl_2$

3mM hexaminecobaltic chloride

Top Agar

0.7% bacto-agar in media (LB or 2x TY) sterilised by autoclaving

solid media melted by microwaving and cooled to 50°C before adding to cells

Trypsin for ES Cell Cultures

Dissolve in 1litre DDW

2.5g trypsin (Difco)

0.4g EDTA

7g NaCl

0.3g $Na_2PO_4.12H_2O$

0.24g KH_2PO_4

0.37g KCl

1.0g glucose

3g Tris

1ml phenol red

adjust pH to 7.6

sterilise by autoclaving

TY (2x)

16g bacto-tryptone

10g bacto-yeast extract

5g NaCl

adjust to pH 7.4 to 1 litre with DDW

APPENDIX II

Sequencing Oligonucleotides

Oligonucleotides listed are shown 5' to 3'

Name	Sequence
seq 1 s	TACAGAGCAACAGTAAAGTCGAGGGGCTG
seq 1 as	CTGGTTCTTCATGGCTGCGGTAGCATTTC
sdm 2 s	TACAGCAGGAGAACAACCACCAGGAAACGT
sdm 2 as	CAATGGATCCCAGGTATTGGTAGGACTGAT
seq 3 s	AACAAGACCCTGCCCATCGCTTTCAAGGT
seq 3 as	TCTTCCGGTGGGGAXATAGAGACCATGAT
seq 4 s	AGAACTTTCCAGTCGACTCTCAACGGCA
seq 5 s	TTCGATATCAAGCTTATCGATACCGTCGAC
seq 5 as	GTCGACGGTATCGATAAGCTT
sdm 2 s	GGCAGGAGAGCACTCGAGTGATCCAGCTG
sdm 2 as	CAGCTGGATCACTCGAGTGCTCTCCTGCC
aml s int	CTACCGCAGCCATGAAGAAACCA
aml as int	GTGGCGACTTGCGGTGGGTTTGTG
aml mid s	GCGCCTTCACCTACTCCCCGACGCCGGTCA
aml mid as	TGACCGGCGTCGGGGAGTAGGTGAAGGCGC
aml 1 as	TAGGAATTCTCAGTAGGGCCTCCACACGG
aml 8 as	CCACCATGGAGAACTGGTAGGAGC
aml end as	GTGGTTCAAGTAAGCCAGGTCCTC
polya s	GAATTCACGCGTGGTACCT
polya as	AGGTACCACGCGTGAATTC
polya 3 s	CGCGGTCCAACAGTAACCATGCTG
polya 3 as	CAGCATGGTTACTGTTGGACCGCG
neo 1 s	GTTTCCACCCAATGTCGAGCA
neo 1 as	TGCTCGACATTGGGTGGAAAC
neo 2 s	GCAGGATCTCCTGTCATCTCA
neo 2 as	TGAGATGACAGGAGATCCTGC

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