Studies on the Core Binding Factor Complex in Patients with Acute Myeloid Leukaemia

Stephen Eirian Langabeer

A thesis submitted to the University of London for the degree of Doctor of Philosophy

2002

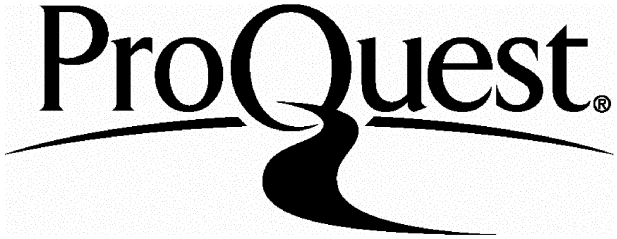
Department of Haematology

University College London

98 Chenies Mews

London

WC1E 6HX



Abstract

The core binding factor (CBF) transcription factor complex is involved in the regulation of a number of genes essential for normal haemopoiesis. The complex is comprised of two subunits: the DNA-binding α subunit AML1 (CBFA2/RUNX1) and the β subunit CBFB, which interacts with AML1 to increase DNA binding. The *AML1* and the *CBFB* genes are known to be disrupted in up to 25% of cases of acute myeloid leukaemia (AML). Therefore, in this thesis, several aspects of CBF leukaemia were investigated at the DNA and RNA level. The chromosomal abnormalities t(8;21)(q22;q22) and inv(16)(p13q22) result in the creation of *AML1-ETO* and *CBFB-MYH11* transcriptionally active fusion genes, and are associated with the M2 and M4Eo AML FAB types respectively. Detection of these abnormalities at presentation is of clinical importance as these patients have a relatively favourable prognosis. Of 321 AML patients studied, 21 (6.5%) had inv(16) whereas 10.3% had reverse transcriptase (RT)-PCR evidence of *CBFB-MYH11* fusion transcripts. *AML1-ETO* transcripts were detected in 51/396 (12.9%) of patients studied, although only 32 (8.1%) of the patients had cytogenetic evidence of t(8;21). Both fusion transcripts were demonstrated in AML patients with FAB types other than those with which they are most commonly associated. This suggests that RT-PCR analysis for these fusion transcripts in all new cases of AML would be of diagnostic relevance. Detection of *AML1-ETO* is not only of value in the classification of AML but affords the opportunity to study minimal residual disease which may aid in determining a patients' response to therapy or may indicate impending relapse. The novel, rapid, RNA based transcription mediated amplification and hybridisation protection assay (TMA/HPA) were evaluated for this purpose. Probes and primers optimised for the TMA/HPA analysis of *BCR-ABL* transcripts were initially available and were evaluated for quantification of these transcripts in chronic myeloid leukaemia patients. *ABL* transcripts were simultaneously quantified and used to control for template RNA quality and assay standardisation. Oligonucleotides were then developed for the quantification of *AML1-ETO* transcripts and TMA/HPA methodology was subsequently applied to samples from AML patients with t(8;21). TMA/HPA was able to identify *AML1-ETO* positive samples at a sensitivity of <1 in 10^4 cells at diagnosis and retrospectively demonstrate changes in the *AML1-ETO/ABL* ratio which reflected patient status or treatment, including prediction of relapse. Mutations in the DNA binding runt domain of *AML1* have been reported in AML patients with the minimally differentiated FAB type M0 and in the rare familial platelet disorder with predisposition to AML (FPD/AML). PCR-single stranded conformation polymorphism (SSCP) analysis was used to screen this region for mutations in DNA samples from 41 M0 and 20 FAB type M7 (megakaryoblastic) AML patients. Two polymorphisms and six mutations were identified. Three mutations would be predicted to result in a truncated protein and 3 would alter AML1 conformation. The incidence of mutations in M0 AML patients (12%) was lower than that previously reported in 2 other similar series (24% and 33%). In addition, a germline mutation of *AML1* was identified in two members of a family with FPD/AML. These studies illustrate the importance of AML1 in both normal haemopoiesis and myeloid leukaemogenesis and identify several molecular abnormalities of the CBF complex which may have a potential impact on clinical management of AML patients.

Acknowledgements

For Mum, Dad and Lisa

I really would like to thank everyone in the Department of Haematology, University College London for help, ideas, encouragement, support etc etc over recent years. I am especially indebted to Professor David Linch for providing me with the opportunity to do this work and to Dr Rosemary Gale, supervisor extraordinaire, whose unrelenting enthusiasm and patience when faced with the candidates' occasional inability to read or count, deserves much praise. I must also thank Mrs Helen Walker and the cytogenetics/DNA bank team in the Department of Haematology, University College Hospital, London and finally Mr Neil Porter and staff, Department of Haematology, Royal Hallamshire Hospital, Sheffield for the introduction to haematology.

Table of contents

		Page
Title		**1**
Abstract		**2**
Acknowledgements		**3**
Table of contents		**4**
List of Figures		**8**
List of Tables		**9**
Abbreviations		**10**
Chapter 1	**Introduction**	**12**
1.1	**Haemopoiesis**	**13**
1.1.1	Haemopoiesis	**13**
1.1.2	Transcriptional control of haemopoiesis	**15**
1.2	**Acute myeloid leukaemia**	**16**
1.2.1	Incidence and aetiology of AML	**16**
1.2.2	Diagnosis of AML	**18**
1.2.3	Treatment of AML	**21**
1.2.4	Cytogenetics of AML	**25**
1.2.5	Molecular genetics of AML	**30**
1.3	**Core binding factor complex**	**33**
1.3.1	Introduction	**33**
1.3.2	*AML1* structure	**34**
1.3.3	*CBFB* structure	**38**
1.3.4	Normal function of CBF	**39**
1.3.5	Disruption of CBF in leukaemia	**44**
1.4	**Aims**	**52**
Chapter 2	**Materials and methods**	**53**
2.1	**General materials and reagents**	**54**
2.2	**Methods**	**56**
2.2.1	Extraction of DNA from PB or BM	**56**
2.2.2	Extraction of DNA from PB or BM smears	**56**
2.2.3	Extraction of RNA from PB or BM	**57**
2.2.4	Agarose gel electrophoresis	**58**
2.2.5	Reverse transcription	**58**
2.2.6	Polymerase chain reaction	**59**
2.2.7	Single strand conformation polymorphism analysis	**59**
2.2.8	End-labelling of primers	**60**

2.2.9	Sequencing of PCR products	**60**
2.2.10	Cloning of PCR products	**62**
2.2.11	Mutation specific enzyme digests	**62**
Chapter 3	**RT-PCR detection of *CBFB-MYH11* in AML**	**64**
3.1	**Introduction**	**65**
3.2	**Materials and methods**	**69**
3.2.1	Patients	**69**
3.2.2	Cytogenetics	**69**
3.2.3	Sample preparation	**70**
3.2.4	RT-PCR analysis	**70**
3.2.5	Sensitivity	**73**
3.2.6	Sequencing	**73**
3.2.7	Restriction enzyme digest	**74**
3.3	**Results**	**74**
3.3.1	Sensitivity	**74**
3.3.2	Incidence of inv(16) by cytogenetics	**77**
3.3.3	Incidence of *CBFB-MYH11* fusion transcripts	**77**
3.3.4	Specificity of RT-PCR products	**77**
3.4	**Discussion**	**79**
Chapter 4	**RT-PCR detection of *AML1-ETO* in AML**	**83**
4.1	**Introduction**	**84**
4.2	**Materials and methods**	**87**
4.2.1	Patients	**87**
4.2.2	Cytogenetics	**88**
4.2.3	Sample preparation	**88**
4.2.4	RT-PCR analysis	**88**
4.2.5	Sensitivity	**91**
4.2.6	Sequencing	**91**
4.3	**Results**	**92**
4.3.1	Sensitivity	**92**
4.3.2	Incidence of t(8;21) by cytogenetics	**92**
4.3.3	Incidence of *AML1-ETO* fusion transcripts	**92**
4.3.4	Specificity of RT-PCR products	**95**
4.4	**Discussion**	**95**
Chapter 5	**TMA/HPA analysis of *BCR-ABL* in CML**	**100**

5.1	**Introduction**	**101**
5.1.1	Introduction	**101**
5.1.2	CML	**101**
5.2	**Principles of TMA and HPA**	**106**
5.2.1	Transcription mediated amplification	**106**
5.2.2	Hybridisation protection assay	**106**
5.3	**Materials and methods**	**109**
5.3.1	Patients	**109**
5.3.2	Cytogenetics	**109**
5.3.3	Sample preparation	**109**
5.3.4	RT-PCR analysis	**109**
5.3.5	TMA	**111**
5.3.6	HPA	**111**
5.3.7	Statistical analysis	**112**
5.4	**Results**	**112**
5.4.1	Technical evaluation of TMA/HPA	**112**
5.4.1.1	Sensitivity and linearity	**112**
5.4.1.2	Reaction kinetics and product stability	**113**
5.4.1.3	Reproducibility	**113**
5.4.2	Analysis of CML patient samples	**116**
5.4.2.1	Diagnosis	**116**
5.4.2.2	Post alloBMT	**118**
5.5	**Discussion**	**121**
Chapter 6	**TMA/HPA analysis of *AML1-ETO* in AML**	**125**
6.1	**Introduction**	**126**
6.2	**Materials and methods**	**128**
6.2.1	Patients	**128**
6.2.2	Sample preparation	**128**
6.2.3	RT-PCR analysis	**129**
6.2.4	TMA/HPA analysis	**129**
6.2.5	Sensitivity	**129**
6.2.6	Reproducibility	**130**
6.2.7	Statistical analysis	**130**
6.3	**Results**	**130**
6.3.1	Technical evaluation of TMA/HPA	**130**
6.3.1.1	Sensitivity and linearity	**130**
6.3.1.2	Reproducibility	**132**
6.3.2	Analysis of AML patient samples	**132**
6.3.2.1	Evaluation of RNA integrity	**132**
6.3.2.2	Diagnosis	**135**

6.3.2.3	During treatment	**137**
6.4	**Discussion**	**137**
Chapter 7	**Mutational analysis of *AML1* in AML**	**143**
7.1	**Introduction**	**144**
7.2	**Materials and methods**	**147**
7.2.1	Patient samples	**147**
7.2.2	PCR-SSCP	**148**
7.2.3	Sequencing	**148**
7.2.4	Cloning	**148**
7.2.5	Mutation specific enzyme digests	**148**
7.2.6	Loss of heterozygosity analysis	**150**
7.3	**Results**	**150**
7.3.1	Mutation detection	**150**
7.3.2	Analysis of samples from patients in haematological remission and relapse and haematologically normal controls	**157**
7.3.3	LOH analysis	**157**
7.4	**Discussion**	**159**
Chapter 8	**Mutational analysis of *AML1* in FPD/AML**	**166**
8.1	**Introduction**	**167**
8.2	**Materials and methods**	**170**
8.2.1	Patients	**170**
8.2.2	Samples	**172**
8.2.3	PCR analysis an sequencing	**172**
8.3	**Results**	**173**
8.4	**Discussion**	**173**
Chapter 9	**Conclusions**	**178**
9.1	**Conclusions**	**179**
9.2	**Future directions**	**184**
References		**187**
Publications arising from work in this thesis		**215**

List of figures

		Page
1.1	Cytokine regulation of haemopoiesis	**14**
1.2	Organisation of the AML1 protein	**36**
1.3	Genomic structure and alternative splicing of the *AML1* gene	**37**
1.4	Genomic structure and alternative splicing of the *CBFB* gene	**40**
1.5	CBF transcriptional complex	**42**
1.6	Alterations of the CBF complex in leukemia	**45**
1.7	ETO family interactions	**47**
1.8	Transcriptional repression mechanism of AML1-ETO	**49**
3.1	*CBFB-MYH11* fusion transcripts	**67**
3.2	*CBFB-MYH11* fusion transcript with primer positions	**72**
3.3	Mispriming of *CBFB* primer	**75**
3.4	Sensitivity of *CBFB-MYH11* RT-PCR	**76**
3.5	Restriction enzyme digest of *CBFB-MYH11* RT-PCR products	**76**
4.1	*AML1-ETO* fusion transcript with primer positions	**90**
4.2	Sensitivity of *AML1-ETO* RT-PCR	**93**
5.1	Genomic structure of *BCR*, *ABL* and *BCR-ABL* genes	**103**
5.2	Transcription mediated amplification	**107**
5.3	Hybridisation protection assay	**108**
5.4	Linearity and sensitivity of *BCR-ABL* and *ABL* TMA/HPA assays	**114**
5.5	Kinetics and reproducibility of *BCR-ABL* and *ABL* TMA/HPA assays	**115**
5.6	*BCR-ABL* and *ABL* levels of CML patients and HNC	**117**
5.7	*BCR-ABL* levels of CML patients post alloBMT and DLI	**119**
6.1	Linearity and sensitivity of *AML1-ETO* and *ABL* TMA/HPA assays	**131**
6.2	Reproducibility of *AML1-ETO* TMA/HPA assay	**133**
6.3	Evaluation of RNA integrity	**134**
6.4	*AML1-ETO* and ABL levels of AML patients and HNC	**136**
6.5	*AML1-ETO* levels of AML patients throughout treatment	**138**
7.1	Conformation of the AML1 runt domain	**145**
7.2	PCR-SSCP patterns of patients with mutations of *AML1*	**151**
7.3	Alternative splicing due to mutation of the *AML1* gene	**154**
7.4	Confirmation of mutations of the *AML1* gene	**156**
7.5	LOH analysis of chromosome 21q22	**158**
7.6	Mutations of the *AML1* gene predicted to result in truncation	**163**
7.7	Mutations of the *AML1* gene predicted to result in conformational change	**164**
8.1	FPD/AML pedigree	**171**
8.2	Characterisation of FPD/AML *AML1* gene mutation	**174**

List of tables

		Page
1.1	Categories of genes targeted by mutations in AML	**19**
1.2	FAB classification of AML	**22**
1.3	WHO classification of AML	**23**
1.4	Incidence of recurrent chromosomal abnormalities in AML	**26**
1.5	Outcome of AML patients defined by cytogenetic risk group	**28**
1.6	AML translocations disrupting transcription factor encoding genes	**31**
1.7	*CBF* and *ETO* gene nomenclature	**35**
3.1	Primers for the detection of *CBFB-MYH11* and *ABL* transcripts	**71**
3.2	Screening for inv(16) and *CBFB-MYH11* transcripts	**78**
4.1	Primers for the detection of *AML1-ETO* transcripts	**89**
4.2	Screening for t(8;21) and *AML1-ETO* transcripts	**94**
5.1	Primers and probes for the RT-PCR and TMA/HPA detection of *BCR-ABL* and *ABL* transcripts.	**110**
7.1	Primers for PCR-SSCP and restriction enzyme digest analysis of *AML1* and LOH analysis	**149**
7.2	Patient characteristics and details of *AML1* mutations	**152**
7.3	Follow up and LOH analysis of patients with *AML1* mutations	**155**

Abbreviations

AE	acridinium ester
ALL	acute lymphoblastic leukaemia
alloBMT	allogeneic bone marrow transplantation
AML	acute myeloid leukaemia
ATP	adenosine triphophate
autoBMT	autologous bone marrow transplantation
bp	base pair
BM	bone marrow
CBF	core binding factor
CD	cluster of differentiation antigen
cDNA	complementary deoxyribonucleic acid
CML	chronic myeloid leukaemia
CP	chronic phase
CR	complete remission
CV	coefficient of variation
DLI	donor lymphocyte infusion
DNA	deoxyribonucleic acid
dNTP	deoxy nucleotide triphosphate
DTAB	dodecyl trimethyl ammonium bromide
ET	essential thrombocythaemia
FAB	French American British
FPD/AML	familial platelet disorder with predisposition to acute myeloid leukaemia
FISH	fluorescence *in situ* hybridisation
G-CSF	granulocyte-colony stimulating factor
GTC-ME	guanidinium thiocyanate-mercaptoethanol
GVHD	graft versus host disease
GVL	graft versus leukaemia
HAT	histone acetyl transferase
HDAC	histone deacetylase
HLA	human leucocyte antigen
HNC	haematologically normal control
HPA	hybridisation protection assay
IL-n	interleukin-n
ISCN	International Standardisation of Cytogenetic Nomenclatue
kb	kilobase
kDa	kilodalton
LOH	loss of heterozygosity
M	molar
MDS	myelodysplastic syndrome

MNC	mononuclear cells
MPD	myeloproliferative disorder
MRC	Medical Research Council
MRD	minimal residual disease
mRNA	messenger ribonucleic acid
NASBA	nucleic acid sequence based amplification
OS	overall survival
PB	peripheral blood
PBS	phosphate buffered saline
PBSC	peripheral blood stem cells
PCR	polymerase chain reaction
RARS	refractory anaemia with ringed sideroblasts
REL	relapse
RLU	relative light unit
RNA	ribonucleic acic
RNApol	ribonucleic acid polymerase
RR	relapse risk
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
SCT	stem cell transplantation
SSCP	single stranded conformation polymorphism
Taq	Taq DNA polymerase
TBE	tris-borate EDTA buffer
TMA	transcription mediated amplification
TSG	tumour suppressor gene

Chapter 1

Introduction

1.1.1 Haemopoiesis

Haemopoiesis is the process by which primitive cells proliferate and differentiate to produce mature blood cells. The circulating blood cells in adult life are derived from a small pool of multipotential haemopoietic cells, primarily located in the bone marrow, known as stem cells. The various blood cells have differing, finite life spans and therefore need to be continuously replenished by a high production rate from the stem cell pool. The need for particular blood cells in the peripheral circulation may vary suddenly, e.g. requirement for neutrophils during acute bacterial infection or erythrocytes to correct anaemia following blood loss, and the bone marrow system has the ability to adapt and respond to these stimuli to increase production rate of particular, functional, end-stage haemopoietic cells as required. This balance is achieved via the regulated systems of cell proliferation, commitment, maturation, survival and removal. In order to produce fully differentiated mature haemopoietic cell populations, progeny of pluripotent stem cells become progressively more committed to specific lineages: an early step appearing to be differentiation commitment to either the lymphoid or myeloid lineages. The myeloid pathway gives rise to granulocytes, erythrocytes, macrophages and megakaryocytes, hence these early progenitors are termed CFU-GEMM, whereas stem cells that give rise to cells of the lymphoid lineage are termed CFU-L (Figure 1.1). These committed progenitors are restricted to the variety of cells they can produce, and the further along the differentiation pathway the cells proceed, the more mature and morphologically, immunologically and functionally distinct the granulocytes (neutrophils, eosinophils, basophils), monocytes/macrophages, erythrocytes, platelets, T and B lymphocytes appear. During this maturation process, an expansion of numbers occurs through cell division to produce the magnitude of end-stage cells of the haemopoietic system.

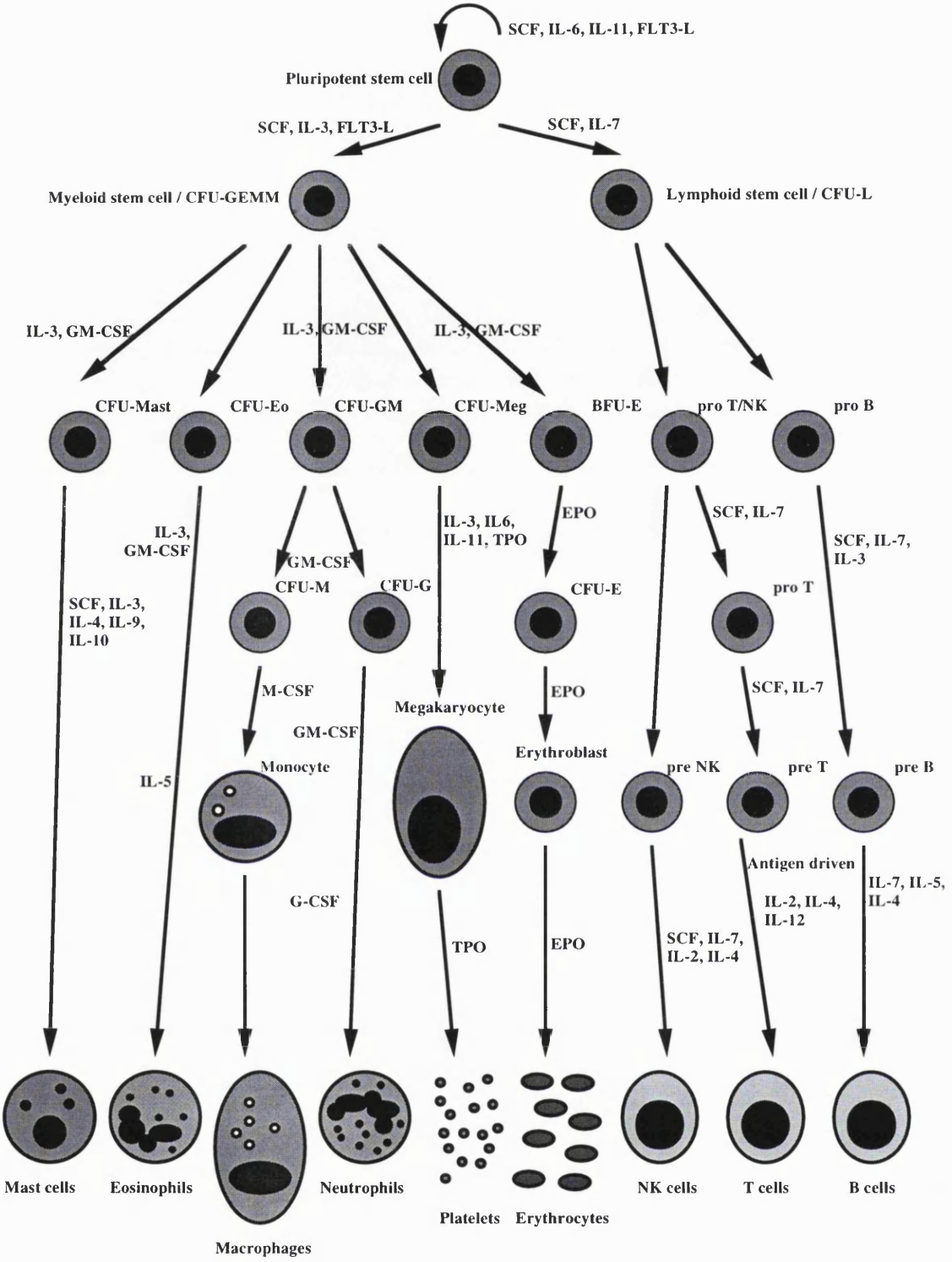


Figure 1.1 Cytokine regulation of haemopoiesis. Pluripotent stem cells in the bone marrow differentiate into various lineages of mature haemopoietic cells. Important cytokines involved in their proliferation and differentiation are indicated. Adapted from Hara & Miyajima (1999). CFU: colony forming unit; BFU: burst forming unit; Eo: eosinophil; GM: granulocyte macrophage; Meg: megakaryocyte; E: erythroid; NK: natural killer; SCF: stem cell factor; FLT3-L: fms like tyrosine kinase-3 ligand; IL-: interleukin-; GM-CSF: granulocyte macrophage-colony stimulating factor; G-CSF: granulocyte-colony stimulating factor; M-CSF: macrophage-colony stimulating factor; TPO: thrombopoietin; EPO: erythropoietin

Regulatory molecules have been characterised that interact with, co-ordinate and stimulate the proliferation of progenitor cells and their progeny, and which initiate maturation events necessary to produce fully mature cells (Metcalf, 1998). These haemopoietic growth factors and cytokines act on one or more lineage, while some exhibit synergistic actions (Figure 1.1). Key regulators for the generation of progenitor cells from stem cells are stem cell factor (SCF) and fms-like tyrosine kinase-3 ligand (FLT3-L). Differentiation from stem cells requires simultaneous stimulation by several regulators, and it has been shown that the interleukins -3, -6, -7, -11 (IL-3, IL-6, IL-7, IL-11) and the granulocyte and granulocyte-macrophage colony stimulating factors (G-CSF, GM-CSF) are active partners in SCF-mediated stimulation. Cytokines such as IL-3, G-CSF and thrombopoietin (TPO) are active both in early and late stages of haemopoiesis. The key regulators for development of mature granulocytes and monocytes are colony-stimulating factors such as G-CSF, GM-CSF, macrophage-colony stimulating factor (M-CSF) and IL-3. Regulators such as G-CSF, M-CSF, erythropoietin (EPO) and TPO have important actions in terms of commitment to a specific lineage: G-CSF is specific for promoting neutrophil production; M-CSF for promoting monocyte and macrophage production; EPO for erythrocyte production; TPO for platelet production (Fraser *et al*, 1996). Bone marrow stromal cells provide a suitable microenvironment to regulate the haemopoietic processes by cell-to-cell interactions and/or by secreting the regulatory cytokines and growth factors (Torok-Storb *et al*, 1999).

1.1.2 Transcriptional control of haemopoiesis

Transcription factors play major roles in haemopoietic development and are critical for myeloid differentiation. These DNA binding molecules interact with gene promoter sequences and regulate mRNA expression. A number of transcription factors have been identified through their involvement in myeloid leukaemias, either

as a result of abnormal expression e.g. PU.1 and C/EBPα (Tenen *et al*, 1997) or through their involvement at the site of recurring chromosomal translocations e.g. AML1, EVI1 and PLZF (Rabbitts, 1994; Look, 1997). These studies have become increasingly important in that acute myeloid leukaemia (AML) results from a block in normal myeloid differentiation, and many of the abnormalities critical to development of myeloid leukaemia involve transcription factors.

Myelopoiesis can be described in a way which emphasises the critical role of these factors. Factors such as PU.1 and C/EBPα are expressed at low levels in myeloid stem cells (Cheng *et al*, 1996). Signals, possibly stromal interactions or growth factor signalling via intracellular signalling pathways, enhance the expression or transcriptional activity of PU.1 or C/EBPα. Activated PU.1 results in increased expression of the M-CSF receptor whereas activated C/EBPα increases the expression of the G-CSF receptor. Increased expression of these receptors allows cells to respond to the appropriate cytokine, resulting in increased proliferation, differentiation and suppression of apoptosis of the monocytic or granulocytic lineages (Behre *et al*, 1999).

1.2 Acute myeloid leukaemia (AML)

1.2.1 Incidence and aetiology of AML

AML is the most common form of acute leukaemia in adults. In the United States, the annual incidence of AML is approximately 2.4 new cases per 100,000 individuals and it increases progressively with age to a peak of 12.6 per 100,000 adults of 65 years of age or older (Kosary *et al*, 1995).

Acute leukaemia, like other cancers, is a progressive clonal disorder driven by mutations. An extraordinary diversity of chromosomal and molecular changes occurs in leukaemic cells, and the restriction of most of these changes to the leukaemic clone suggests that these are acquired not inherited. However, a small proportion (up to 5%) of both acute myeloid and lymphoid leukaemias are associated with inherited, predisposing genetic syndromes, often involving genes that encode proteins with functions related to genomic stability and DNA repair (Horwitz *et al*, 1997). Different biological subtypes of leukaemia may have distinct causal mechanisms, and there may be functional links between particular molecular abnormalities and causal agents. Aetiological mechanisms involved in producing the initiating mutation may differ from those inducing or promoting subsequent mutations, and the time span covering relevant exposure, sequential mutations and clinical diagnosis of the disease may be very long.

Although the cause of most types of acute leukaemia is unknown, certain major factors have been implicated in some cases (Greaves, 1997). Firstly, the best substantiated causal mechanism for acute leukaemia is due to ionising radiation. Much of the current understanding has been based on documentation of 1945 atomic bomb survivors in Japan in whom there is a substantially increased incidence of both chronic and acute myeloid and lymphoid malignancies (Preston *et al*, 1994). Leukaemia has also been associated with occupational exposures to ionising radiation, e.g. in health workers, nuclear processing plant workers and airline pilots (Gundestrup & Storm, 1999; Schubauer-Berigan & Wenzl, 2001). Cohorts of patients who have received radiotherapy for malignant conditions have also been found to be at an increased risk for leukaemia (Dann & Rowe, 2001), particularly AML. Secondly, an infectious cause of some cases of human acute leukaemia has been suggested as most spontaneous leukaemias in domesticated animals are viral in origin. Evidence of a viral aetiology of human leukaemia comes from the role of Epstein-Barr virus in

Burkitt's lymphoma (Niedobitek *et al*, 2001) and human T cell lymphotropic virus-type 1 in adult T cell leukaemia (Siegel *et al*, 2001). Thirdly, certain chemicals, especially organic solvents (e.g. benzene), can contribute to myeloid leukaemogenesis (Wong, 1995). Many agents used in chemotherapy such as alkylating agents or DNA topoisomerase II inhibitors are genotoxic and induce DNA strand breaks. Such therapy related malignancies are predominantly AML (Dann & Rowe, 2001).

Genes involved in the pathogenesis of AML are thought to act by two general mechanisms. The first involves the structural alteration of a normal gene whose protein product induces malignancy (oncogenes). This altered protein is usually involved in cellular proliferation, differentiation or survival. The second mechanism involves loss or inactivation of genes whose proteins suppress malignant transformation. Genes of this class are known as tumour suppressor genes (Cline, 1994). Alterations by mutation, fusion, rearrangement or loss in members of specific gene groups are consistently associated with AML (Table 1.1). Certain oncogenes are activated in a wide variety of malignancies, whereas others are restricted to haemopoietic myeloid tissues and leukaemias. These can be broadly grouped into categories which consist of genes that encode intracellular signalling molecules, transcription factors, or are involved in differentiation, apoptosis and suppression of malignancy.

1.2.2 Diagnosis of AML

AML is a malignant clonal disorder of immature haemopoietic cells characterised by aberrant cellular proliferation and maturation. A variety of subtypes of human AML exist, characterised by their commitment to a particular lineage and by the extent of their differentiation. In order for a definitive diagnosis of AML to be made, at least 30% of the bone marrow nucleated cells must be myeloblasts. Before initiation of

Table 1.1 Categories of genes targeted by mutations in AML.

Type of cell component disrupted	Example
Cell surface receptors	*FLT3*, *C-KIT*
Intracellular signalling molecules	*RAS*
Nuclear membrane proteins	*NUP98*
Nuclear receptors	*RARα*
Transcription factors	*AML1*, *C/EBPα*
Transcription factor coordinators	*MLL*, *CBP*, *MOZ*
DNA repair enzymes	*MSH2*
Carcinogen metabolising enzymes	*GSTT*, *MTHFR*, *NQO1*
Cell cycle control proteins	*RB*, *P53*

chemotherapy, AML must be distinguished from acute lymphoblastic leukaemia (ALL), myelodysplastic syndromes (MDS) and AML arising from MDS, as treatment strategies for these diseases vary considerably.

The initial diagnosis of AML rests on the morphological and cytochemical identification of myeloblasts in bone marrow and peripheral blood stained smears. AML blast cells are large and uniform in size, have finely dispersed chromatin, prominent nucleoli and a high nucleus to cytoplasm ratio. The cytoplasm frequently contains fine granules and a variable number of Auer rods. The morphological features may be equivocal and so cytochemical stains can be useful in characterising subtypes of AML and also delineating poorly differentiated AML from ALL. In the majority of cases of AML, a variable proportion of the blasts are positive for myeloperoxidase, non-specific esterase and periodic acid Schiff stains. However, there remains a significant minority of cases that cannot be characterised by these methods and these may be more accurately characterised by immunophenotyping techniques such as immunohistochemical staining or by flow cytometry. The lineage of haemopoietic cells is defined both by the expression and absence of antigens associated with a particular lineage. Panels consisting of an array of antibodies to myeloid and lymphoid associated antigens are used for immunophenotyping which contain antibodies to CD13 or CD33 (myeloid markers), CD2 or CD3 (T lymphoid), CD19 or CD20 (pan B lymphoid), CD4 and CD8 (lymphoid), CD14 (monocytic), CD34 (primitive), glycophorin A (erythroid) and CD61 (megakaryocytic). However, leukaemic blasts may aberrantly express some antigens of another lineage or lack expression of an expected antigen.

Once a diagnosis of AML is made, the morphological and cytogenetic subtype of AML must be determined. The most commonly used classification is the French-American-British (FAB) system which divides AML into a minimum of 9 distinct

subtypes (Bennett *et al*, 1976, 1985a, 1985b, 1991) defined by morphology and reactivity with cytochemical stains (Table 1.2). Distinct cytogenetic abnormalities are often seen in AML and are associated with specific FAB types, e.g. t(15;17) associated M3 and inv(16) associated M4Eo. Several cytogenetic lesions can also be used to identify subgroups of patients with distinct clinical features and therapeutic responses (see Chapter 1.2.4). More recently, a combination of morphological, immunophenotypical, cytogenetic and clinical features has been used to define distinct disease entities in the WHO classification of AML (Harris *et al*, 1999) (Table 1.3). Thus cytogenetic and molecular methods have become increasingly used in the diagnosis of patients with AML.

1.2.3 Treatment of AML

The primary objective in treating patients with AML is to induce a remission and subsequently prevent relapse. Complete remission (CR) is conventionally defined morphologically by myeloblasts accounting for fewer than 5% of non-erythroid cells in the bone marrow, together with the recovery of peripheral blood counts towards normal levels. More sensitive immunological and molecular methodologies are now available which are able to characterise remission status more accurately. The patient's age is crucial in relation to treatment in AML. The likelihood of achieving initial disease remission and of remaining in CR declines with increasing age. The approach to therapy differs according to whether the patient is aged above or below about 60 years of age. About 40% of patients under 60 can survive beyond 5 years, compared with only approximately 10-15% of patients over 60 years of age (Lowenberg *et al*, 1999).

Treatment for AML can be conventionally divided into 2 phases: induction and post induction. For the last 3 decades, treatments to induce remission in AML have

Table 1.2 The French-American-British (FAB) classification of AML. Adapted from Bain (1998).

FAB subtype	Common name (% of all AML cases)	Bone marrow characteristics
M0	Acute myeloblastic leukaemia with minimal differentiation (<5%)	MPO positive in <3% blasts, blasts demonstrated to be myeloid by immunophenotyping
M1	Acute myeloblastic leukaemia without maturation (15%)	Blasts >90% NEC, >3% blasts positive for MPO, monocytic component <10% NEC, granulocytic component <10% NEC
M2	Acute myeloblastic leukaemia with maturation (20-25%)	Blasts 30-90% NEC, granulocytic component >10% NEC, monocytic component <20% NEC
M3	Acute promyelocytic leukaemia (10%)	Characteristic heavily granulated blasts
M4	Acute myelomonocytic leukaemia (20%)	Monocytic component >20% NEC, cytochemical confirmation of monocyte component, PB monocytes >5x10^9/l
M4Eo	Acute myelomonocytic leukaemia with abnormal eosinophils (<10%)	As M4 with >5% abnormal eosinophils
M5	Acute monocytic leukaemia (10%)	With/without monocytic maturation, monocytic component >80% NEC
M6	Acute erythroblastic leukaemia (<5%)	Erythroblasts >50% blasts >30% NEC
M7	Acute megakaryoblastic leukaemia (<10%)	Blasts demonstrated to be megakaryocytic by cytochemistry or immunophenotyping

MPO: myeloperoxidase; NEC: non-erythroid cells.

Table 1.3 World Health Organisation (WHO) classification of AML. Adapted from Harris *et al* (1999). MDS: myelodysplastic syndrome.

AML with recurrent cytogenetic translocations
- with t(8;21)(q22;q22), *AML1(CBFα)/ETO*
- acute promyelocytic leukaemia (AML with t(15;17)(q22;q11-12) and variants, *PML/RARα*)
- with abnormal bone marrow eosinophils (inv(16)(p13q22) or t(16;16)(p13;q22), *CBFβ/MYH11*)
- with 11q23 (*MLL*) abnormalities

AML with multilineage dysplasia
- with prior MDS
- without prior MDS

AML and MDS, therapy-related
- alkylating agent related
- epipodophyllotoxin-related
- other types

AML not otherwise categorised
- AML minimally differentiated
- AML without maturation
- AML with maturation
- acute myelomonocytic leukaemia
- acute monocytic leukaemia
- acute erythroid leukaemia
- acute megakaryocytic leukaemia
- acute basophilic leukaemia
- acute panmyelosis with myelofibrosis

Acute biphenotypic leukaemia

---

included the nucleoside analogue cytarabine and the anthracycline daunorubicin. Other anthracyclines known to be effective in AML include idarubicin and mitoxantrone. For those induction regimens that contain daunorubicin and cytarabine, CR can be induced in 70-80% of patients less than 60 years of age and in approximately 50% of older patients (Lowenberg *et al*, 1999).

Once remission is achieved, further intensive treatment of AML patients is necessary to prevent relapse. Three options are available to younger patients: allogeneic bone marrow/stem cell transplantation (alloBMT/SCT) from an HLA-matched related or unrelated donor, autologous BMT/SCT (autoBMT/SCT), or further consolidation chemotherapy. AutoBMT involves the administration of higher doses of chemotherapy, but is limited by the lack of the graft versus leukaemia effect associated with alloBMT. Furthermore, there is a theoretical risk of infusion of residual leukaemic cells. AlloBMT provides the best anti-leukaemic potential, but it is consistently associated with a higher risk of treatment-related mortality than the other two strategies. High dose chemotherapy may be of value, but this approach may only be tolerated by younger patients. Overall, maximum intensity treatment in the consolidation phase is likely to prevent relapse in younger AML patients. However, whatever the treatment approach, outcome will be highly influenced by prognostic risk factors. In younger patients, age, karyotype at diagnosis and the ability to eliminate myeloblasts from the bone marrow with the initial treatment are the most powerful determinants of relapse and hence survival (Burnett, 2001).

Most patients presenting with AML are over 60 years of age and many of these patients are not offered intensive treatments as they are not as well tolerated as in younger patients. Older patients with AML frequently have poor prognosis karyotypes (see Chapter 1.2.4) and many have leukaemic cells that express proteins, such as p-glycoprotein, that render the cells resistant to chemotherapy (Sonneveld &

List, 2001). Those who are generally well are offered induction chemotherapy followed by low dose maintenance, but this is still associated, even with good prognostic indicators, with a long term survival of less than 20% (Buchner *et al*, 2001). Many older patients are now considered for more experimental therapeutic approaches.

Bacterial and fungal infections during or after induction therapy are common in AML patients, thus the use of haemopoietic growth factors to accelerate recovery and prevent infection is possible. G-CSF and GM-CSF have been shown to shorten the period of neutropenia after induction (Estey, 2001), but their role in increasing response to chemotherapy and prolonging survival remains to be established.

Acute promyelocytic leukaemia (APL), is characterised by the t(15;17) translocation which results in the disruption of a gene encoding a retinoic acid receptor. The effectiveness of the vitamin A derivative all-*trans* retinoic acid (ATRA) as a differentiating agent in combination with chemotherapy in patients with this disease has been confirmed in clinical trials (Burnett *et al*, 1999).

Novel approaches to AML treatment include anti-CD33-drug chelated antibodies (Sievers *et al*, 2001), toxin-growth factor conjugates to specifically target myeloid cells (Hall *et al*, 2001), and the induction of leukaemia antigen specific cytotoxic T cells by dendritic cells (Claxton & Choudhury, 2001).

1.2.4 Cytogenetics of AML

During the last three decades it has become apparent that many cases of AML are characterised by at least one of a variety of recurrent chromosomal abnormalities (Table 1.4). The significance of these abnormalities is twofold. Firstly, cytogenetic

Table 1.4 Recurrent chromosomal abnormalities in AML patients entered into the UK MRC AML 10 trial. Adapted from Grimwade *et al* (1998).

Abnormality	Incidence (% of total)
No abnormality	42%
t(15;17)	12%
+8	9%
t(8;21)	8%
Complex	6%
-7	4%
11q23	4%
inv(16)	4%
+21	3%
Abnormal (3q)	3%
del(7q)	2%
del(5q)	2%
-5	2%
del(9q)	2%
+22	1%
Other numerical	14%
Other structural	23%

Complex karyotype defined by the presence of at least five unrelated abnormalities

analysis has significantly increased the understanding of the genetic basis of AML, leading to the mapping and cloning of genes whose disruption or deregulation contributes to the leukaemogenic process. Secondly, chromosomal abnormalities have been shown to be markers of great diagnostic and prognostic value (Table 1.5). In the new WHO classification of haematological malignancies (Harris *et al*, 1999), some of these specific cytogenetic abnormalities have been used to help define distinct disease entities of AML (Table.1.3).

The majority of AML patients with successful cytogenetic examination at diagnosis display acquired clonal chromosomal abnormalities i.e. an identical structural aberration, gain or loss of the same chromosome in at least three metaphases (Mitelman, 1995). In studies of adults with *de novo* AML, abnormal karyotypes have been detected in 40-75% of patients (Keating *et al*, 1988; Fenaux *et al*, 1989; Schiffer *et al*, 1989; Dastugue *et al*, 1995, Bloomfield *et al*, 1998; Grimwade *et al*, 1998). The reasons for this variation may be attributed to advances in cytogenetic technologies and the increased level of awareness of subtle structural aberrations such as the t(11;19), t(15;17) or the inv(16) which may sometimes be overlooked, particularly in preparations of suboptimal quality. The absence of chromosomal alterations in a proportion of patients with AML appears to be a genuine phenomenon. Spectral karyotyping (SKY) and fluorescence *in situ* hybridisation (FISH) techniques, which enable the simultaneous visualisation of all chromosomes in different colours, have confirmed that gross chromosomal rearrangements are absent in a number of AML patients (Veldman *et al*, 1997; Mohr *et al*, 2000). The presence of an exclusively normal karyotype at diagnosis, however, does not mean that the myeloblasts do not harbour any genetic alterations beyond the resolution of these techniques.

Acquired chromosomal abnormalities have been reported in thousands of patients world-wide. Although these data are very heterogenous, with almost any kind of

Table 1.5 Outcome of AML patients defined by cytogenetic risk group. Adapted from Grimwade *et al* (1998).

Risk group	Abnormality	CR rate (%)	RR*(%)	OS*(%)
Favourable	t(8;21)	98	29	66
	t(15;17)	87	37	63
	inv(16)	88	42	61
Intermediate	No abnormality	88	53	42
	+8	84	44	48
	11q23	87	47	45
	+21	80	50	47
	del(7q)	75	59	23
	del(9q)	100	39	60
	+22	91	51	59
	Other numerical	76	60	29
	Other structural	76	51	35
Adverse	Complex	67	68	21
	-7	54	80	10
	Abnormal (3q)	63	85	12
	del(5q)	57	85	11
	-5	42	90	4

CR: complete remission; RR: relapse risk; OS: overall survival; *: at 5 years. Patients with del(9q) and +22 were included in the intermediate group, despite their relatively favourable outcome, due to their frequent association with t(8;21) and inv(16) respectively. Similarly, cases with del(7q) or other structural and numerical changes were also included in the intermediate group, although they exhibited a poorer outcome than patients with normal cytogenetics due to an association with abnormalities within the adverse risk category. However, in the absence of associated favourable or adverse cytogenetic features, the outcome of patients with del(9q), +22, del(7q), or those with other structural or numerical abnormalities did not differ significantly from patients with normal cytogenetics.

structural or numerical abnormality described, the distribution of chromosome aberrations is highly non-random. Certain chromosomes, chromosome regions and bands are involved in AML-associated changes more frequently than others, e.g. 3q26, 21q22, 11p15 (Mrozek *et al*, 2001a). Primary chromosomal abnormalities, often observed alone, are important as disruption of the genes involved is considered to be a critical step in leukaemogenesis. Approximately 45% of all AML patients with a cytogenetically abnormal clone have two or more chromosomal rearrangements in the karyotype. These secondary abnormalities, observed in addition to primary changes, may be present at diagnosis or relapse and their type seems to depend to some degree on the primary aberration (Johansson *et al*, 1994) such as +22 with inv(16) and loss of a sex chromosome with t(8;21).

Studies on large numbers of patients over the past fifteen years have established that the karyotype at diagnosis represents one of the most important, independent, prognostic determinants in patients with AML, with significant differences in CR, relapse risk (RR) and overall survival (OS) (Keating *et al*, 1988; Fenaux *et al*, 1989; Schiffer *et al*, 1989; Dastugue *et al*, 1995, Bloomfield *et al*, 1998; Grimwade *et al*, 1998). Those abnormalities in patients with a relatively favourable overall survival are the t(15;17) associated with APL (Burnett *et al*, 1999, Avvisati *et al*, 2001), and the t(8;21) and inv(16) that disrupt genes which encode members of the core binding factor (CBF) transcription factor complex (Bloomfield *et al*, 1998, Grimwade *et al*, 1998; Slovak *et al*, 2000). Abnormalities associated with a relatively poor prognosis include a complex karyotype, and translocations and deletions involving chromosomes 3, 5 and 7 (Dastugue *et al*, 1995; Grimwade *et al*, 1998). All other abnormalities tend to be associated with a relatively intermediate prognosis (Table 1.5). Cytogenetic analysis can therefore serve to identify biologically distinct subsets of AML that required tailored therapeutic approaches. Those patients with abnormalities associated with prolonged survival are treated with intensive

chemotherapy and are spared high risk transplantation procedures, whereas those patients with chromosomal abnormalities associated with poor outcomes are considered for more experimental therapeutic approaches. This has led towards routine cytogenetic characterisation of newly diagnosed AML, which currently provides a framework for treatment stratification.

1.2.5 Molecular genetics of AML

Elucidation of the molecular genetic basis of leukaemias has derived from cloning and characterisation of the acquired mutations in haemopoietic progenitor cells. Clues to the localisation of possible leukaemogenic genes have been provided by the aforementioned recurring chromosomal abnormalities that can be identified by conventional cytogenetic techniques. It is now possible to condense the numerous and diverse array of abnormalities seen in patients with AML into related groups based on similar structure and function of the genes involved in these abnormalities (Dash & Gilliland, 2001). A common theme in AML associated with balanced reciprocal translocations is the generation of a chimaeric product resulting in aberrant function of a transcription factor. These transcription factors are frequently conserved in evolution, and are important in both human development as well as in normal haemopoiesis. Transcription factors involved in AML include CBFs, retinoic acid receptor alpha (RARα), homeobox (HOX) family members and also transcriptional co-repressors and co-activators (Table 1.6).

The t(15;17) translocation is associated with the clinical type of APL and results in the creation of a *PML-RARα* fusion gene. This encodes a protein containing functional RAR and DNA binding domains from PML (Fenaux *et al*, 1997) which acts as a dominant negative inhibitor of the wild type PML protein. At the cellular level, the vitamin A derivative ATRA, acts as ligand for RAR and induces

Table 1.6 AML associated translocations resulting in disruption of normal transcription factor function.

Translocation	Fusion gene
A. Core binding factor	
t(8;21)	*AML1-ETO*
t(3;21)	*AML1-MDS1/EVI1*
t(16;21)	*AML1-MTG16*
inv(16)	*CBFB-MYH11*
B. Retinoic acid receptor	
t(15;17)	*PML-RARα*
t(11;17)	*PLZF-RARα*
t(5;17)	*NPM-RARα*
C. HOX family members	
t(7;11)	*NUP98-HOXA9*
t(2;11)	*NUP98-HOXD13*
t(12;13)	*TEL-CDX2*
t(7;12)	*TEL-HLXB9*
D. ETS family members	
t(12;22)	*MN1-TEL*
t(16;21)	*FUS-ERG*
t(4;12)	*BTL-TEL*
t(1;12)	*TEL-ARNT*

differentiation of the arrested promyelocytes into neutrophils, whereas at the molecular level, ATRA overrides the dominant inhibitory activity of PML-RARα. It has been demonstrated that PML-RARα recruits a nuclear co-repressor that inhibits transactivation from RARα target genes through the recruitment of molecules Sin3A and histone deacetylase (HDAC) (Lin *et al*, 1998). HDACs deacetylate histones and inhibit the binding of transcription factors, thus repressing the expression of genes required for haemopoietic differentiation. The binding of ATRA to the PML-RARα protein results in the release of the nuclear co-repressor complex, and the subsequent expression of genes required for terminal differentiation (Grignani *et al*, 1998).

The HOX family of transcription factors, that regulate embryonic body pattern formation, are involved in lineage determination during early haemopoietic development. Together with upstream regulators, HOX genes are rearranged in a number of translocations observed in patients with AML (Table 1.6). HOX fusion genes are thought to transform haemopoietic progenitor cells through dysregulated overexpression (van Oostveen *et al*, 1999).

The recruitment of coactivators and co-repressors by transcription factors in AML suggests a critical role for these proteins in leukaemogenesis. The translocations t(8;16), inv(8) and t(10;16) rearrange genes encoding modulators and facilitators of gene expression disrupting the *CBP* and *MOZ* genes. CBP fusion proteins may suppress wild type CBP activity, or the transcriptional activation and transformation properties are possibly due to loss of function of one *CBP* allele (Giles *et al*, 1998). MLL is thought to bind to DNA and facilitate binding and recruitment of transcription factors. More than thirty translocations involving different partners of the *MLL* gene have been reported in AML patients (Mrozek *et al*, 2001a). The diversity of partners and the invariable expression of a truncated *MLL* gene product expressed from its

own promoter suggests that a critical aspect of myelopoiesis is associated with *MLL* (Dimartino & Cleary, 1999).

1.3 Core binding factor (CBF) complex

1.3.1 Introduction

The cloning of chromosomal breakpoints associated with particular types of leukaemia has led to the identification of fusion proteins thought to contribute, due to their recurrent detection, to the pathogenesis of these leukaemias. The *AML1* gene is one of the most frequently disrupted by these translocations. The importance of this gene and *CBFB*, which encodes its heterodimeric partner, in normal and malignant haemopoiesis has become apparent by: (1) the identification of these factors as critical regulators of normal myeloid-specific transcription, (2) the involvement of both genes in leukaemia-associated translocations and (3) the failure of haemopoiesis upon deletion of these genes.

The core binding factor (CBF) transcription factor was originally characterised by study of the murine polyoma virus enhancer and the identification of similar sequences in the promoters of haemopoietic genes. The DNA binding factor that recognises these sequences was called polyoma enhancer binding protein 2 (PEBP2) and was found to be composed of two subunits (Kamachi *et al*, 1990). This complex was also known as CBF for its ability to bind a core enhancer sequence, TGTGGT, of murine moloney leukaemia virus (Speck *et al*, 1992). In humans, CBFs contain one subunit that binds to DNA directly (CBFA), encoded by one of three genes (*CBFA1/AML3*, *CBFA2/AML1*, *CBFA3/AML2*) and a second, non-DNA binding subunit, CBFB, encoded by the *CBFB* gene. Molecular cloning of the human homologues of the murine CBF genes and assignment by the Human Genome

Organisation has yielded competing and potentially confusing nomenclature which is summarised in Table 1.7. For the remainder of this thesis, the AML1/CBFB nomenclature is employed.

1.3.2 *AML1* structure

The *AML1* gene is located on chromosome 21q22.2 and is a member of a family of genes all with homology to the Drosophila *runt* gene (Levanon *et al*, 1994). *AML3* (*CBFA1*) has been shown to be required for the generation of osteoblasts in mouse and human bone (Komori *et al*, 1997; Otto *et al*, 1997) and is the gene mutated in the human genetic disease cleidocranial dysplasia which is characterised by multiple bony abnormalities (Lee *et al*, 1997; Mundlos *et al*, 1997). *AML2* has not yet been associated with any disease but its expression appears to be under control of retinoid nuclear receptors and it may play a role in haemopoietic cell differentiation (Le *et al*, 1999). These three genes are all expressed, but not exclusively, in haemopoietic cells (Table 1.7).

The *AML1* gene spans 260 kb of genomic DNA and its expression is regulated through two distinct promoter regions (Ghozi *et al*, 1996). Two major functional domains have been identified: (1) the 118 amino acid, N-terminal, *runt* homology domain encoded by exons 3-5, which is involved in DNA and heterodimer binding, and (2) the transactivation domain encoded by exons 6-8 which contains multiple co-repressor and co-activator protein binding sites (Friedman, 1999) (Figure 1.2). Twelve transcripts are produced due to alternative splicing of exons (Miyoshi *et al*, 1995; Levanon *et al*, 1996; Hattori *et al*, 2001) and are predicted to give rise to various proteins. AML1b (453 amino acids) is the major protein isoform consistently detected in cells which contains the DNA binding and transactivation domains (Figure 1.3). The minor AML1c protein differs from AML1b only in the N terminal region (480

Table 1.7 Nomenclature of *CBF* and *ETO* genes.

Acute myeloid leukaemia (human)	Polyoma virus enhancer (murine)	Retroviral core (murine)	Other	HUGO*	Chromosomal localisation	Disease association	Expression pattern
AML1	*PEBP2αB*	*CBFA2*		*RUNX1*	21q22	AML, ALL, MDS	Haemopoietic but widely distibuted
AML2	*PEBP2αC*	*CBFA3*		*RUNX3*	1p36	None to date	Haemopoietic cells
AML3	*PEBP2αA*	*CBFA1*	*OSF2*	*RUNX2*	6p12	CCD**	Predominantly bone
CBFB	*CBFβ*	*CBFB*		*CBFB*	16q22	AML	Widely distributed
ETO	-	-	*MTG8*	*CBFAT1*	8q22	AML	Brain, some haemopoietic cells
-	-	-	*MTGR1*	*CBFAT2*	20q11	None to date	Widespread
ETO2	-	-	*MTGR2, MTG16*	*CBFAT3*	16q24	AML, MDS	Widespread

*HUGO: Human Genome Organisation; **CCD: cleidocranial dysplasia.

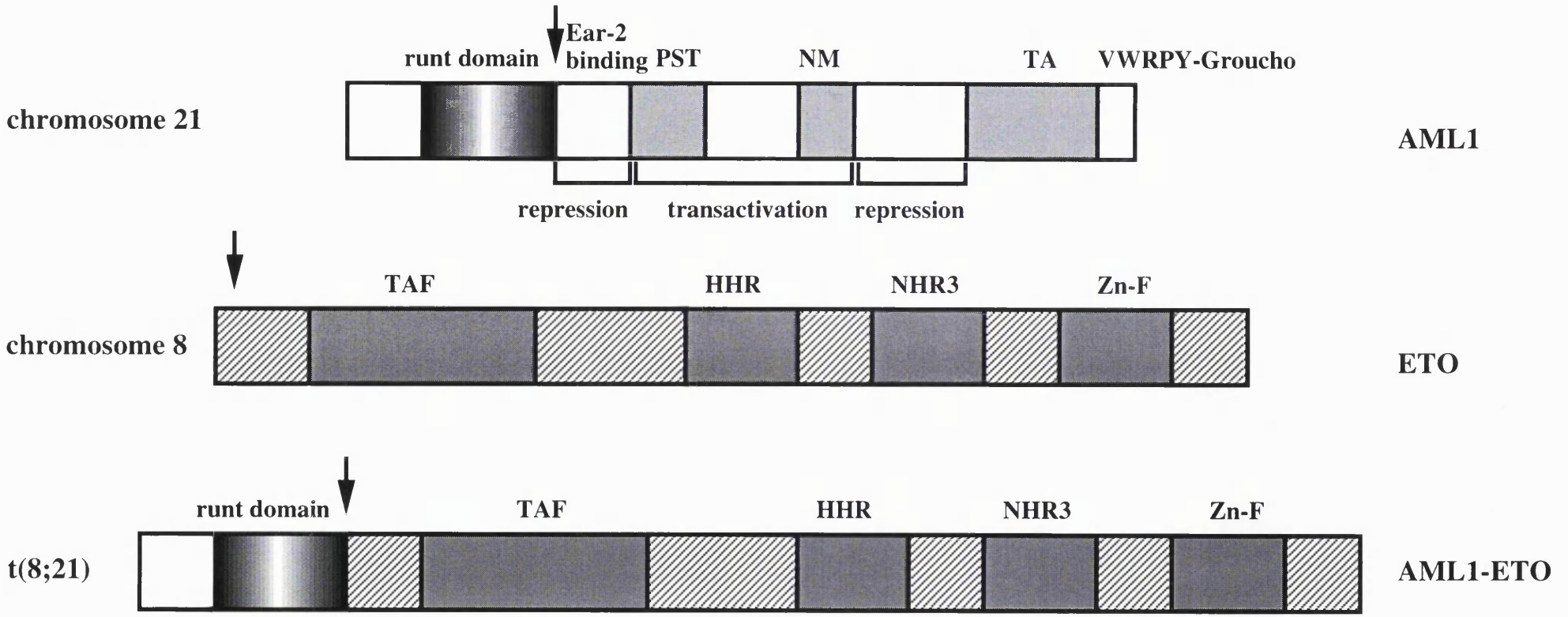


Figure 1.2 The AML1 protein is organised into a runt domain, a proline/serine/threonine-rich region (PST), a nuclear matrix attachment sequence (NM) and a transcriptional activation domain (TA). A further two repression domains exist, one of which interacts with the Ear-2 protein. AML1 ends in the amino acid sequence VWRPY which binds to the Groucho co-repressor. The ETO protein contains four regions with homology to *Drosophila* Nervy (grey): a transcription-activating factor domain (TAF), a hydrophobic heptad repeat (HHR), a region referred to as the Nervy homology region 3 (NHR3) and a domain that contains two Zn-finger (Zn-F) motifs. The structure of the AML1-ETO product is illustrated with chromosome breakpoints indicated by arrows.

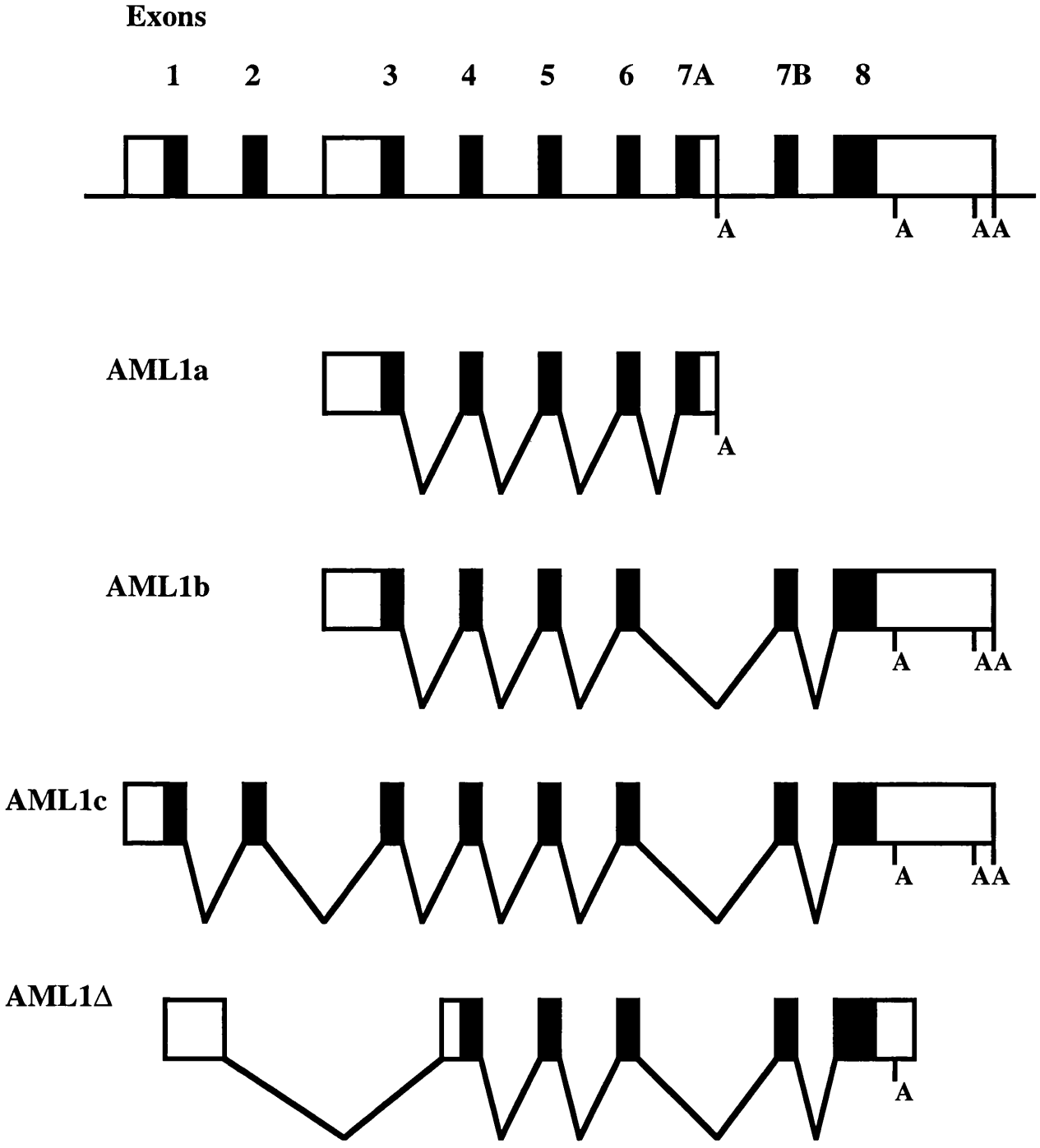


Figure 1.3 Schematic representation of alternative splicing of *AML1* transcripts. A schematic representation of the exons is shown at the top. Solid boxes represent the coding regions and open boxes represent the 5' and 3' untranslated regions. Positions of polyadenylation signals are shown by A. Adapted from Miyoshi *et al* (1995).

amino acids) and probably has the same function (Miyoshi *et al*, 1995). AML1a is present at low levels in normal bone marrow cells, lacks the transactivation domain (Figure 1.3) but is nevertheless capable of suppressing AML1b function (Tanaka *et al*, 1995). AML1Δ, which represents a relatively small fraction of total *AML1* transcripts, does not bind DNA as it does not possess the entire runt domain and also interferes with AML1b transactivation (Zhang *et al*, 1997). *AML1* is expressed in haemopoietic cell lines such as HL60 and K562, in T and B lymphoid cell lines, and is highly expressed in lymphoid tissues. Among human tissues, *AML1* is predominantly expressed in haemopoietic cells but is evident in virtually all tissues except the heart and the brain (Levanon *et al*, 1994; Miyoshi *et al*, 1995).

The primary sequence of the *AML1* runt domain shows no homology to any of the known DNA-binding motifs. A predicted model of the structure of the runt domain was described (Levanon *et al*, 1998) and was subsequently determined to be an S-type immunoglobulin fold (Nagata *et al*, 1999; Berardi *et al*, 1999) which displayed similarities to the core DNA binding domains of other transcription factors. Further analysis of the runt domain-CBFB-DNA complex has identified the surfaces of AML1 which are in contact with either DNA (Huang *et al*, 1999) or CBFB (Warren *et al*, 2000) and have suggested an enhanced DNA clamping mechanism on CBFB heterodimerisation (Bravo *et al*, 2001). Functional studies of disease related, runt domain amino acid AML1 mutants have provided molecular evidence that the primary defect is a failure of DNA recognition (Nagata & Werner, 2001; Tahirov *et al*, 2001).

1.3.3 *CBFB* structure

CBFB was first characterised in mice by its involvement as a cofactor of AML1 (Ogawa *et al*, 1993). Only a single gene encoding CBFB has been identified in

mammals, unlike the AML1 subunit that consists of a family of three related genes. The human *CBFB* gene maps to chromosome 16q22 and contains six exons that are alternatively spliced to yield four protein isoforms (Figure 1.4) (Adya *et al*, 2000). CBFB 187 and 182 isoforms are generated by two different splice donors at the 3' end of exon 5. CBFB 148 and 155 isoforms are generated by splicing out of exons 3 and 5 respectively (Figure 1.4). *CBFB* has a long 3' untranslated region that may have a role in mRNA degradation. Human and mouse *CBFB* genes are extremely homologous to each other and the mRNA appears to be expressed in all adult human tissues analysed and in cell lines derived from mouse and human tissues. CBFB is predominantly present in the cytoplasm, whereas AML1 is always in the nucleus. The main role of CBFB proteins is to enhance the DNA binding ability of AML1. Although AML1 binds DNA very weakly, its affinity for the consensus sequence is enhanced fivefold when it is complexed with CBFB (Huang *et al*, 1998). The stoichiometry of the two subunits in the CBF complex is 1:1. The CBFB 182 and 187 isoforms interact with AML1 similarly, whereas the 148 and 155 isoforms do not stably associate (Ogawa *et al*, 1993).

The N-terminal amino acids of CBFB contain the heterodimerisation domain for DNA-binding AML1 subunits which is sufficient for CBFB function in vivo (Huang *et al*, 1998). Analysis of the high resolution structure of CBFB reveals a novel α/β structure with a distinct heterodimerisation surface (Huang *et al*, 1999).

1.3.4 Normal function of CBF

The CBF complex has been shown to function as a transcriptional activator that is critical for the tissue-specific expression of a number of haemopoietic specific genes including myeloperoxidase, the M-CSF receptor, subunits of the T cell antigen receptor (TCRα, β and δ), neutrophil elastase, neutrophil protein-3 and the cytokines

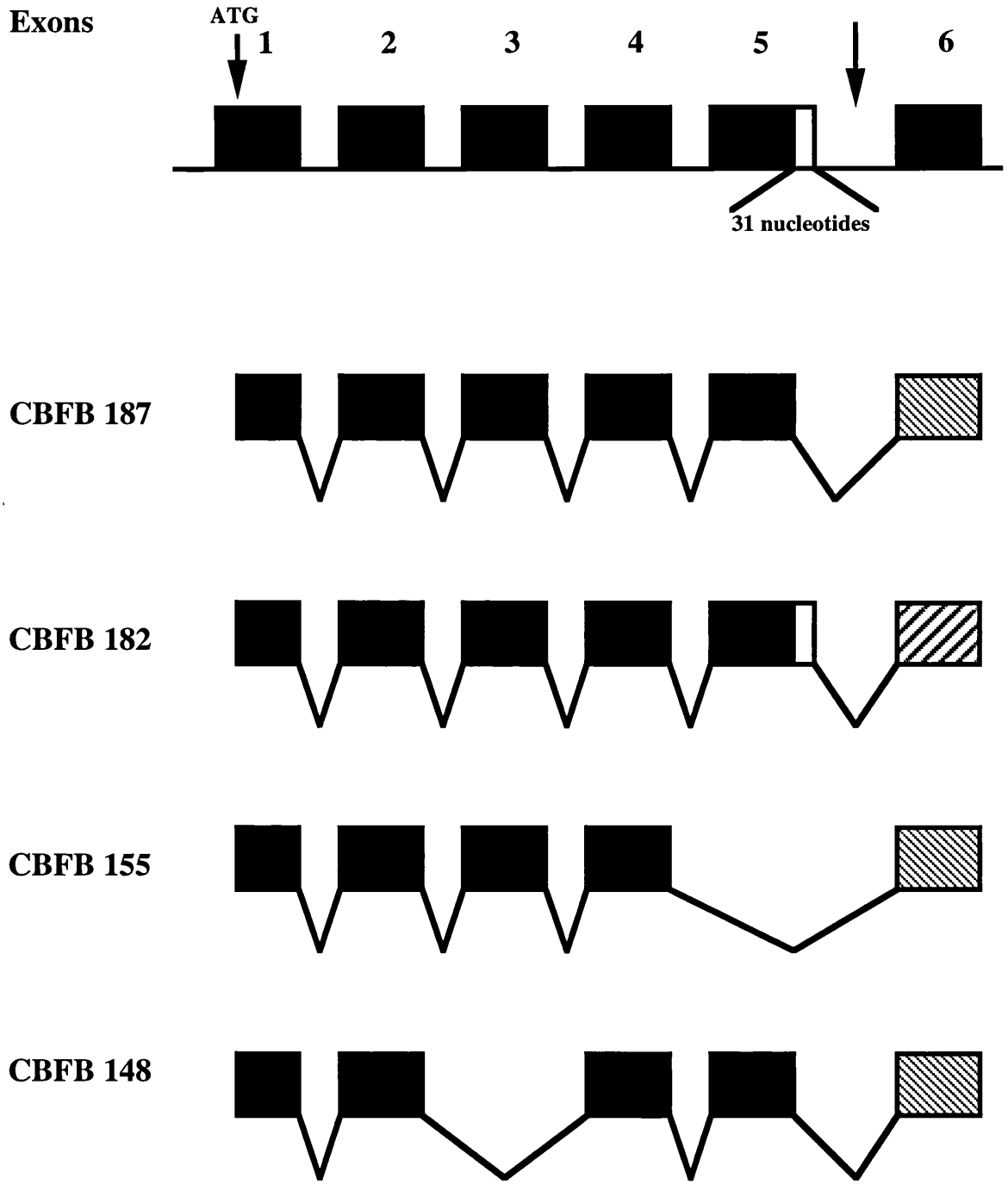


Figure 1.4 Genomic structure and alternative splicing products of the *CBFB* gene. The two different amino acid sequences encoded by exon 6, generated by alternative splice donor sites in exon 5 that are 31bp apart, are indicated by different shadings. The locations of almost all inv(16) breakpoints (see Figure 3.1) are in intron 5 indicated by an arrow. Adapted from Adya *et al* (2000).

IL-3 and GM-CSF (Licht, 2001). Although binding of AML1/CBFB to the core enhancer sequence, TGTGGT, is important for expression of these genes, their expression is also dependent on the presence of adjacent binding sites for lineage-restricted transcription factors such as C-MYB, C/EBPα and ETS family members (Downing, 1999). This observation has led to the suggestion that the CBF complex functions as a transcriptional organiser that recruits tissue-specific factors into a nucleoprotein complex (Figure 1.5).

In addition, AML1 is also influenced by the adjacent binding of sequence-specific DNA-binding proteins such as LEF-1, which is facilitated by the coactivator ALY, which directly binds to both proteins (Bruhn *et al*, 1997). AML1-mediated transcriptional activation also involves the direct binding of the coactivators p300 and CBP to the transcriptional activation domains of AML1 (Kitabayashi *et al*, 1998a). These coactivators have intrinsic histone acetyltransferase (HAT) activity and induce the acetylation of lysine residues in chromatin associated histones, resulting in a change in the chromatin structure that leads to enhanced transcription (Redner *et al*, 1999).

AML1 contains several other functional motifs that are important for its biological activity. These include a proline/serine/threonine-rich region (PST) which contains potential phosphorylation sites for kinase pathways, a nuclear matrix attachment site (NM), a second transcriptional activation domain and two transcriptional repression domains, one of which binds Ear-2 (Figure 1.2). The C-terminal VWRPY amino acids of AML1 function as specific binding sites for the transcriptional co-repressor Groucho. Binding of Ear-2 (Ahn *et al*, 1998) or Groucho (Aronson *et al*, 1997) proteins to the CBF complex results in conversion of its activity to that of a transcriptional repressor.

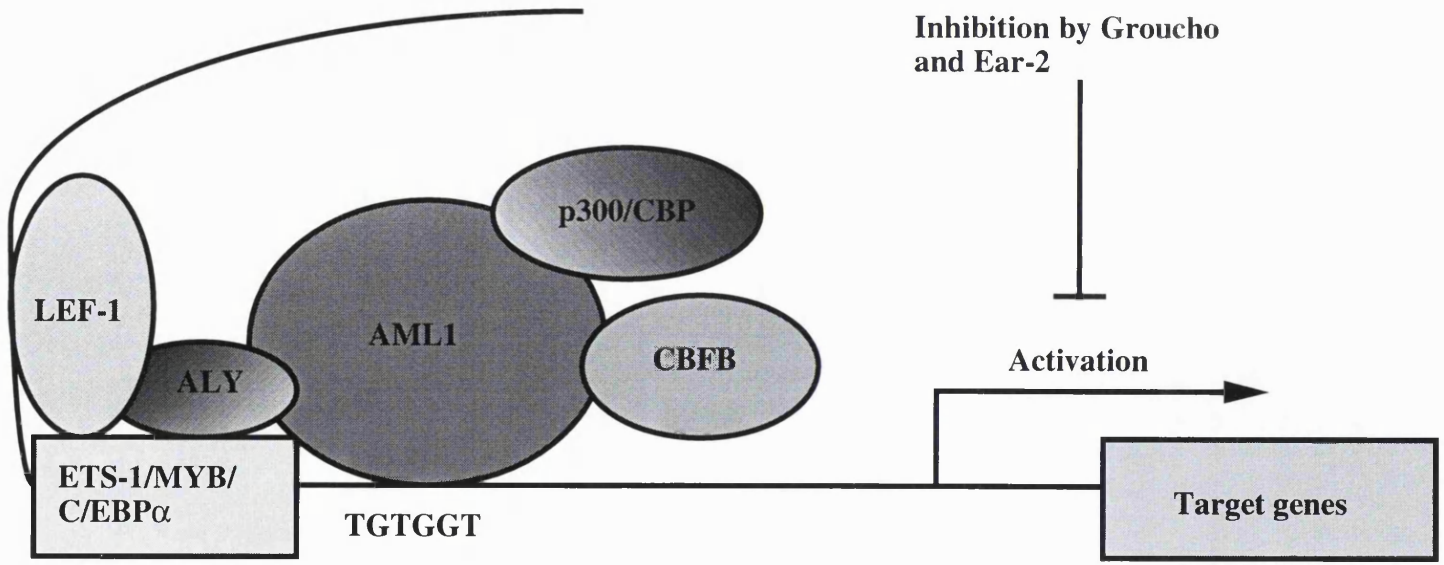


Figure 1.5 The CBF complex functions as an enhancer organising factor to induce gene transcription via recruitment of co-activators.

Although the CBF complex appears to function normally as a transcriptional activator, alternatively spliced forms of AML1 have been identified that lack sequences encoding either the transcriptional activation domain (Miyoshi *et al*, 1995) or the runt domain (Zhang *et al*, 1997) (Figure 1.3). These transcripts are normally present at low levels but alterations in the minor:major isotype ratios have been shown to result in transcriptional repression in myeloid cells by blocking granulocytic differentiation in response to G-CSF (Tanaka *et al*, 1995a; Zhang *et al*, 1997).

These data suggest that expression of the CBF complex could lead to either transcriptional activation or repression, depending on the specific target genes being regulated, the isoform of AML1 being expressed, and the cellular context in which this occurs. The turnover of transcriptionally competent AML1 has also been recently shown to be controlled by its interaction with CBFB which, when complexed with AML1, inhibits its proteolytic degradation by the ubiquitin-proteasome pathway (Huang *et al*, 2001). Therefore, alterations in the balance of positive and negative signals that are mediated through the CBF complex are likely to contribute directly to haemopoietic cell development and transformation.

Further information of the role of the CBF complex has come from mouse experiments which are consistent with its critical actions in normal haemopoiesis. Gene disruption experiments in mice have shown that both *AML1* and *CBFB* are required for the differentiation of all haemopoietic cells derived from the stem cells (Okuda *et al*, 1996; Wang *et al*, 1996a). In the adult, pluripotent stem cells are the source of all blood cells, however, they are not the first cells to develop in the embryo. The first blood cells appear in the yolk sac and/or the aorta-gonad-mesonephros (AGM) region of mouse embryos at 8 days post coitus (dpc). Yolk sac derived haemopoietic stem cells were thought to give rise to the primitive erythroid lineage and then sequentially migrate (at approximately 10 dpc), expand and

differentiate into the definitive erythrocytes and cells of myeloid lineages in the foetal liver, spleen and bone marrow. However, the first haemopoietic stem cells capable of giving rise to complete engraftment of adult recipients appear in the embryo by the end of 10 dpc within the AGM region (Medvinsky & Dzierak, 1996). The relationship of primitive and definitive AGM stem cells and the migration of these cells through foetal liver before colonisation of the BM remains unclear (Medvinsky & Dzierak, 1999).

Mutations in several genes encoding transcription factors such as *SCL*, *RBTN2*, *GATA1* and *GATA2* have profound effects on the development of both primitive and definitive haemopoiesis (Orkin, 1995) whereas other genes such as *PU.1*, *MYB* and *NF-E2* seem to be required for the development of particular lineages of definitive but not primitive haemopoiesis. In mice, mutations of the *AML1* and *CBFB* genes specifically impair the development of all definitive haemopoietic lineages but not the primitive erythroid lineage (Okuda *et al*, 1996; Wang *et al*, 1996a & b; Sasaki *et al*, 1996). *AML1*- and *CBFB*-deficient embryonic stem cells are unable to contribute to definitive haemopoietic lineages in chimaeric mice, indicating that the block is intrinsic to the stem cells.

1.3.5 Disruption of CBF in leukaemia

Over the past decade it has become increasingly apparent that disruption of the CBF complex, by a variety of mechanisms, is a common finding in a significant proportion of patients with diverse forms of acute leukaemia, particularly AML. The t(8;21) is one of the most common cytogenetic abnormalities seen in patients with AML. Cloning of the t(8;21) and the t(3;21) translocation led to the identification of the *AML1* gene, which is fused to the *ETO* gene on chromosome 8q22 or the *MDS1/EVI1* genes on chromosome 3q26, respectively (Nucifora & Rowley, 1995) (Figure 1.6).

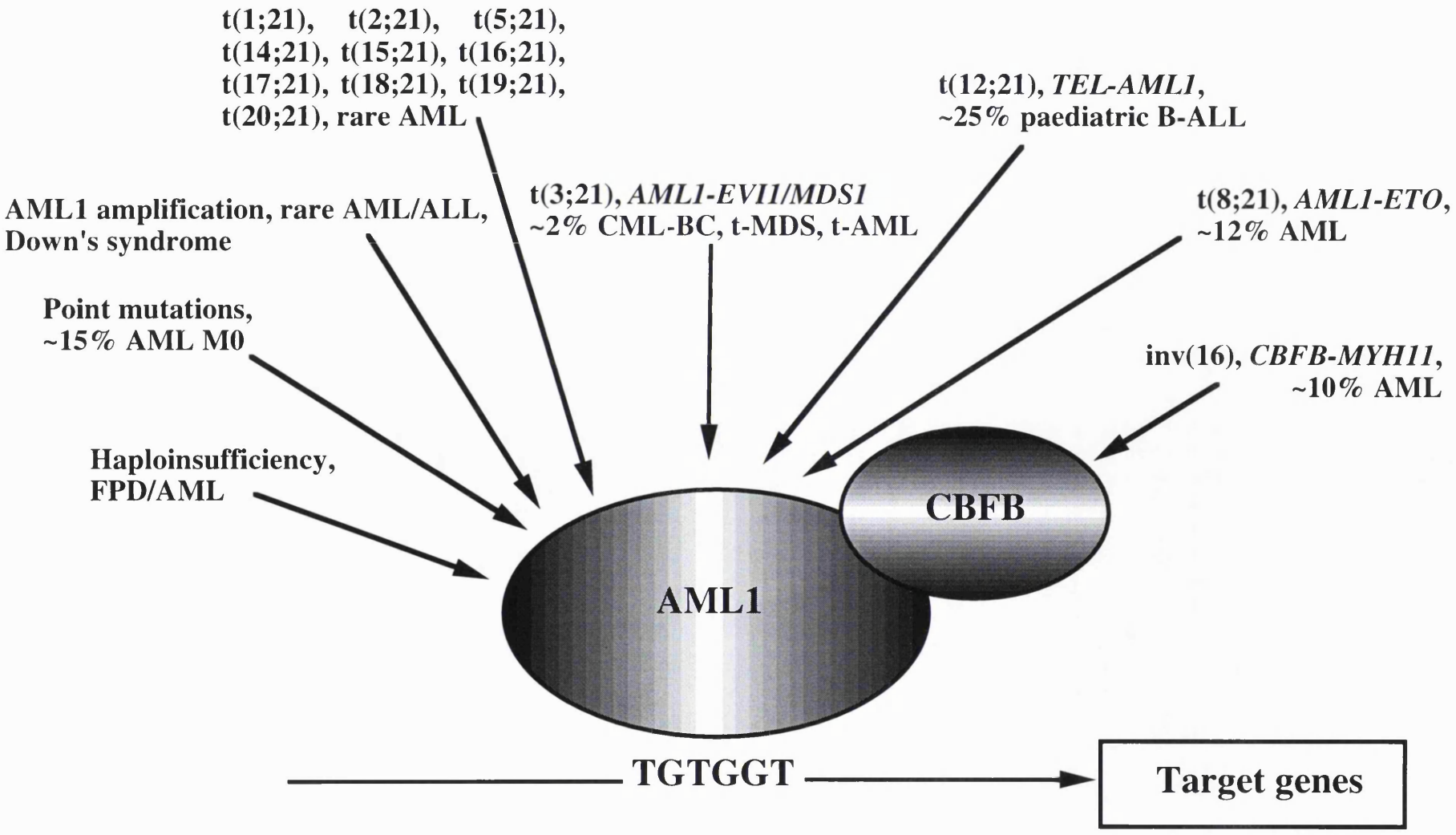


Figure 1.6 Alterations of the CBF complex in acute leukaemia.
BC: blast crisis; t-: therapy related; FPD: familial platelet disorder.

Further studies revealed that the gene encoding the non DNA-binding unit of the CBF complex, *CBFB*, was also disrupted by the inv(16) and t(16;16) cytogenetic abnormalities, resulting in the production of *CBFB-MYH11* fusion transcripts (Liu *et al*, 1995). The application of FISH has increased the resolution of cytogenetic analysis, leading to the detection of an increasing number of translocations that disrupt *AML1* in a variety of leukaemias (Figure 1.6). One of the most striking findings has been the presence of a t(12;21) translocation, resulting in the production of a *TEL-AML1* fusion gene, in approximately 25% of paediatric patients with pre-B ALL (Borkhardt *et al*, 1999). Recent mutational analysis of *AML1* in patients with AML has revealed the existence of insertion, deletion and substitution mutations (Osato *et al*, 1999; Preudhomme *et al*, 2000) which are predicted to alter AML1 function by resulting in either a truncated or structurally altered protein. In addition to physical abnormalities, it is becoming evident that either loss or gain of the complete *AML1* gene may contribute to leukaemogenesis via divergent mechanisms (Barton & Nucifora, 2000; Busson-Le Coniat *et al*, 2001).

In two rare AML translocations, *AML1* is fused to either *MTGR1* or *MTG16* (Table 1.7) (Gamou *et al*, 1998; Kitabayashi *et al*, 1998b). These two genes belong to the *ETO* family as they display considerable sequence homology to *ETO*. This suggests that *ETO* sequences are critical for the transforming properties of these fusion proteins. These ETO family members contain several conserved domains which include a region with homology to transcriptional coactivators of the TAF family, a Nervy homology region 3 (NHR3) and two Zn finger motifs, one of which is termed MYND (Figure 1.2). This structure suggests that ETO is likely to function as a regulator of transcription. ETO can directly interact with the nuclear co-repressors N-CoR and Sin3A and in so doing can recruit an active HDAC (Gelmetti *et al*, 1998; Lutterbach *et al*, 1998) (Figure 1.7). ETO family members have also been shown to

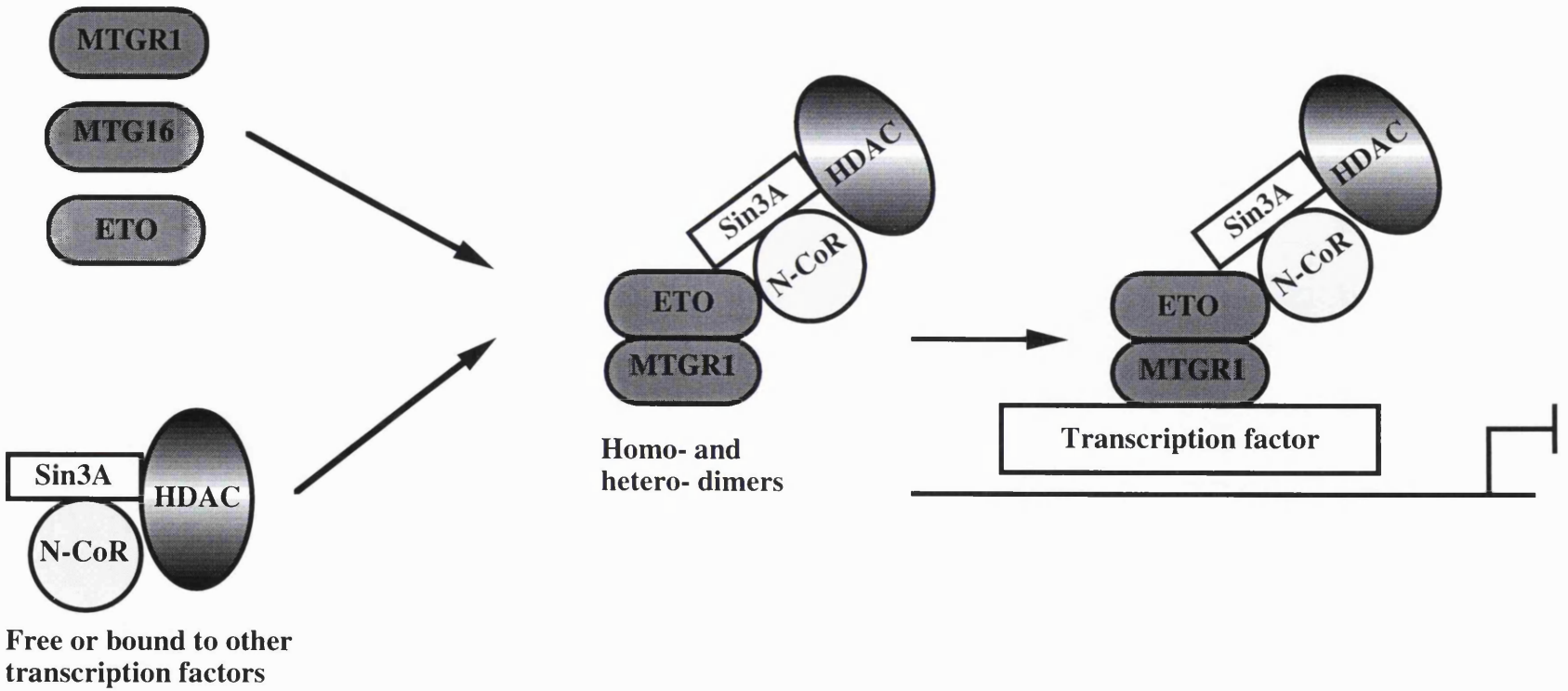


Figure 1.7 Three members of the ETO family can form homo-and hetero-dimers and can interact with nuclear co-repressors. These in turn bind and modify HDAC, inducing a change in chromatin structure that inhibits gene transcription. Adapted from Downing (1999).

dimerise with each other and this may lead to functional differences of the multisubunit complexes that are formed which function in transcriptional regulation.

Thus in t(8;21) AML, AML1-ETO can not only homodimerise, but can also heterodimerise with other ETO family members as the necessary ETO sequences are retained (Figure 1.2). The interaction of AML1-ETO with MTGR1 results in an increase in AML1-ETO mediated transcriptional repression (Kitabayashi *et al*, 1998). These interactions would be predicted not only to alter AML1 function, but also to modify the activities of the interacting ETO family members. In transient transcription assays, AML1-ETO directly represses AML1-mediated transcriptional activation (Meyers *et al*, 1995). This activity of AML1-ETO is dependent on both the runt domain of AML1 and domains within ETO (Lenny *et al*, 1995; Lutterbach *et al*, 1998). Transcriptional repression by AML1-ETO therefore requires direct DNA binding by the fusion protein and is mediated through the nuclear co-repressor complex by ETO (Figure 1.8). AML1-ETO has also been demonstrated to repress the transcriptional activity of C/EBPα and a subset of ETS proteins (Westendorff *et al*, 1998). This activity is mediated by the direct binding of AML1-ETO resulting in changes of chromatin structure by HDAC co-repressors. This transcriptional repression can be partially reversed by inhibitors of HDACs (Wang *et al*, 1999), consistent with the critical role of HDACs in mediating the transcriptional effects of AML1-ETO.

In mice, studies have established that the t(8;21) and inv(16) involving the *AML1* and *CBFB* genes create alleles of these genes that dominantly inhibit wild type AML1 and CBFB function. Two groups (Yergeau *et al*, 1997; Okuda *et al*, 1998) modified *AML1* in such a way that the chimaeric protein created by the t(8;21) was expressed. Using a similar strategy, the inv(16) was created in mice by introducing a cDNA encoding the CBFB-MYH11 fusion protein into the *CBFB* locus (Castilla *et al*, 1996). In all three

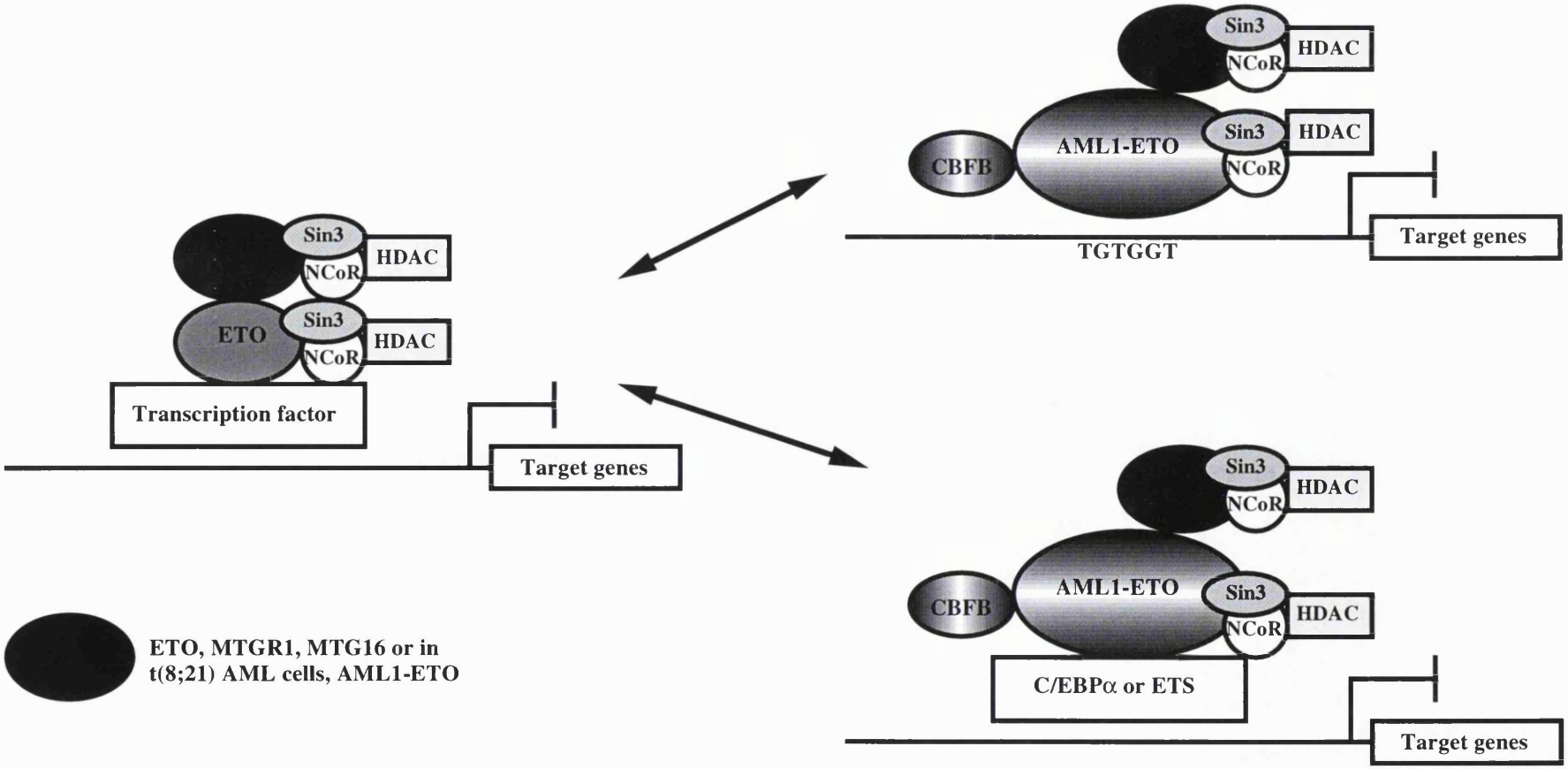


Figure 1.8 ETO forms hetero- and homo-dimers with MTGR1/MTG16 in normal cells and with AML1-ETO in t(8;21) AML cells. The members of the ETO family also interact with nuclear co-repressors Sin3 and NCoR which recruit an active HDAC. ETO family members interact with transcription factors resulting in binding to specific DNA sequences. In t(8;21) AML, AML1-ETO either directly binds the core enhancer sequence or interacts with C/EBPα or ETS to induce transcriptional repression. Adapted from Downing *et al* (2000).

studies, chimaeric mice containing the "knocked-in" fusion genes were created but failed to produce offspring heterozygous for the new allele. The embryos died in midgestation due to central nervous system haemorrhage and exhibited a range of phenotypes similar to those seen in AML1- and CBFB-deficient mice, including a severe impairment of foetal liver haemopoiesis. Mice have now been generated in which the expression of *AML1-ETO* is under control of a tetracycline-inducible system (Rhoades *et al*, 2000). These mice do not develop leukaemia, demonstrating the requirement for additional genetic changes necessary for the transformation to t(8;21) AML. Treatment of these mice with the DNA-alkylating agent N-ethyl-N-nitrosourea (ENU), to induce further mutations, has been recently shown to result in acute leukaemia. Furthermore, ENU treated *CBFB-MYH11* mice develop leukaemia with myelomonocytic features similar to those associated with human inv(16) AML (Castilla *et al*, 1999) (see Chapter 3) whereas the *AML1-ETO* mice develop granulocytic sarcoma and myeloid leukaemia with features reminiscent of human t(8;21) AML (Higuchi *et al*, 2000; Yuan *et al*, 2001) (see Chapter 4).

Studies performed on a number of other AML1 fusion proteins have also provided further insights into the pathogenesis of CBF leukaemia. The t(12;21) encoded TEL-AML1 has been shown to directly function as a constitutive transcriptional repressor in a manner similar to AML1-ETO (Hiebert *et al*, 1996). Repression requires the helix-loop-helix domain of TEL along with the runt domain of AML1 and Sin3 interaction domains, and involves the recruitment of histone deacetylases into the transcriptional complex (Fenrick *et al*, 1999). Although the transcription regulatory domains of AML1 located C-terminal to the runt domain in TEL-AML1 are absent, transcriptional repression is the dominant activity of this fusion protein.

Another AML1 translocation that has been extensively studied is the t(3;21). Although this translocation can theoretically encode three different fusion products,

the critical transforming fusions appear to be *AML1-EVI1* and *AML1-MDS1-EVI1* (Tanaka *et al*, 1995b). Transformation by these products requires both the runt domain of AML1 and the zinc finger domains of EVI1. The latter domains appear to provide transcriptional regulatory functions that include the ability to interact with Smad3, an intracellular mediator of the inhibitory factor transforming growth factor beta (TGFβ), blocking TGFβ-induced growth inhibition of myeloid cells (Kurokawa *et al*, 1998). In contrast to other AML1 fusion proteins, however, AML1-EVI1 does not appear to function by recruiting histone deacetylases to AML1-regulated target genes. Alteration of transcriptional activity may result from competitive inhibition due to direct DNA binding of the fusion protein.

A number of other translocations of chromosome band 21q22 have been reported in secondary AML. These translocations disrupt *AML1* creating novel fusion genes. Partner genes of *AML1* have been located at 1p32, 1p36, 2p11, 5q13, 12q24, 14q22, 15q22, 16q24, 17q11, 18q21, 19q13, 20q11 and 20q13 (Gamou *et al*, 1998; Roulston *et al*, 1998; Calabrese *et al*, 2000; Hromas *et al*, 2000; Richkind *et al*, 2000; Cherry *et al*, 2001). Many of these fusion genes retain the runt domain of *AML1*, emphasising the importance of this region in AML1 function.

Recent data also suggest that overexpression of *AML1* by gene amplification may play a role in leukaemogenesis. Tandem amplifications of the whole *AML1* gene have been described in a number of cases of ALL and AML (Dal Cin *et al*, 2001; Streubel *et al*, 2001). Although the amplified regions are quite large, *AML1* appears to be consistently involved, suggesting it has a functional role. An increased incidence of ALL and AML is seen in patients who have an increased dose of *AML1* as a result of trisomy 21 in Down's syndrome (Taub, 2001). Thus, overexpression of AML1 also appears to functionally alter normal CBF activity, resulting in leukaemia.

1.4 Aims

The aim of the work presented in this thesis was to study several aspects of CBF leukaemia at the DNA and RNA level. Firstly, the incidence and prognostic significance of *CBFB-MYH11* and *AML1-ETO* transcripts was determined. This entailed the use of RT-PCR to screen a large number of patients at presentation of AML. Secondly, the novel transcription-mediated amplification and hybridisation protection assay methodologies, which enable the detection and quantification of specific mRNAs, were evaluated. This system was applied to the detection of *BCR-ABL* transcripts in patients with CML in chronic phase and after alloBMT and was subsequently adapted to retrospectively quantify *AML1-ETO* transcript levels in patients with t(8;21) AML at presentation and follow-up. Thirdly, mutations in the runt domain encoding region of *AML1* have been found in a high proportion of patients with AML M0 and also in FPD/AML. In many cases, these mutations have been described in both alleles of the *AML1* gene. Identification of *AML1* mutations was performed in a large group of AML patients with M0 and M7 FAB subtypes. These mutations were further characterised to assess whether they occurred in one or both alleles of the *AML1* gene. In addition, a family with features of FPD/AML was investigated for the presence of *AML1* mutations.

Chapter 2

Materials and methods

2.1 General materials and reagents

Polypropylene tubes (0.5 / 1.5ml)	Eppendorf
Universal tubes (25ml)	Sterilin
Polypropylene tubes (50ml)	Falcon / Becton Dickinson
Pipettes (5 / 10 / 25ml)	Sterilin
Pipette tips	Gilson / Anachem / Eppendorf / Molecular BioProducts
Polypropylene test tubes	Elkay
Centrifuge tubes (15ml)	Sarstedt
PBS (phosphate buffered saline)	Gibco
Tris (trimethylamine)	BDH / Merck
Orthoboric acid	BDH / Merck
EDTA (ethylenediaminetetra acetic acid-disodium salt)	Sigma
DTAB (dodecyl-trimethyl ammonium bromide)	Sigma
GTC (guanidium thiocyanate)	Sigma
2-ME (mercapto ethanol)	BDH / Merck
Ethanol (AnalaR)	BDH / Merck
Isopropanol (AnalaR)	BDH / Merck
Phenol-water saturated (AnalaR)	BDH / Merck
Chloroform (AnalaR)	BDH / Merck
Na-lauryl-sarcosine (Sarkosyl)	BDH / Merck
Sodium acetate	BDH / Merck
DEPC (diethyl pyrocarbonate)	BDH / Merck
SDS (sodium dodecyl sulphate)	Sigma
Agarose	Sigma

Low Melting Point Agarose	BioWhittaker Molecular Applications
Polyacrylamide	National Diagnostics
TEMED (tetramethylethylenediamine)	Sigma
APS (ammonium persulphate)	Sigma
PCR primers	Oswel
Restriction enzymes / buffers	New England Biolabs

10x TBE Buffer (pH 8.3). For 1L	-Tris	108.9g
	-Orthoboric acid	55.7g
	-EDTA	7.4g
DTAB (8%). For 50ml	- DTAB	4.0g
	- NaCl	4.4g
	- EDTA	0.93g
	- Tris	0.61g
GTC-ME. For 250ml	- GTC	118g
	- 2-ME	1.8ml
	- Na citrate (0.75M)	8.35ml
	- Sarkosyl (20%)	6.25ml

All materials, solutions and disposables used in the extraction of nucleic acids were either sterile, autoclaved or washed in water treated with DEPC (0.1%) to minimise the risk of contamination and degradation by RNAses.

2.2 Methods

2.2.1 Extraction of DNA from PB or BM

Materials DTAB reagent, chloroform, ethanol, water.

Method (Adapted from Gustincich *et al*, 1991). For DNA extraction, BM or PB cells ($10\text{-}100x10^6$) were obtained from buffy coats, washed and resuspended in 2.4ml PBS. DTAB reagent (8%) (4.8ml) was added, mixed thoroughly and incubated at 68°C for 5 minutes. An equal volume of chloroform (7.2ml) was then added to the cells, mixed thoroughly and centrifuged at 3500rpm for 15 minutes. The aqueous layer was decanted into a new 15ml centrifuge tube and the DNA precipitated with an equal volume of chilled ethanol (100%). The sample was then centrifuged at 3000rpm for 5 minutes to pellet the DNA which was transferred to a 1.5ml tube. The DNA was washed in 1ml 75% ethanol, resuspended in an appropriate volume of water, allowed to dissolve thoroughly and stored at 4°C.

2.2.2 Extraction of DNA from PB or BM smears

Materials IGEPAL CA-630 (Sigma), TNE buffer (10mM Tris pH 8.0, 10mM NaCl, 10mM EDTA), SDS (10%), Proteinase K (Sigma), phenol, chloroform, glycogen (Boehringer Mannheim), ethanol, water.

Method (Adapted from Fey *et al,* 1987). Cells were scraped from fixed and stained BM and PB smears with a scalpel blade, collected in a small petri dish and transferred to a 1.5ml tube. They were lysed with 1ml 0.025% IGEPAL for 30 minutes at room temperature and a cell pellet formed by spinning at 13000rpm in a microfuge for 15 minutes. To release DNA, the cell pellet was incubated with 50µl

TNE (x10), 100µl SDS (10%), 100µl Proteinase K (100ng/µl) and 250µl water at 37°C overnight. This was then subjected to a phenol-chloroform extraction (see Chapter 2.4), NaCl added to a final concentration of 0.2M, 2 volumes of ethanol (100%) and 1µl glycogen added and the DNA allowed to precipitate at -20°C overnight. The DNA was subsequently pelleted and washed in ethanol (75%), resuspended in 20-50µl water and stored at 4°C.

2.2.3 Extraction of RNA from PB or BM

Materials GTC-ME, phenol, chloroform, ethanol, DEPC treated water.

Method (Adapted from Chomczynski & Sacchi, 1997). For RNA extraction, BM or PB cells were obtained from buffy coats, washed in PBS and pelleted. One ml GTC-ME was added to cells (5-50x10^6) from BM or PB in PBS and mixed thoroughly. To each 1ml of cells in GTC-ME, phenol (1000µl), chloroform (240µl) and Na acetate (120µl, 3M, pH 4.0) were added and placed on ice for 15 minutes. The samples were then spun at 13000 rpm in a microfuge after which the top aqueous layer (approximately 1ml) was decanted into a new tube. The RNA was the precipitated by addition of 2ml volumes of isopropanol (100%), washed with 75% ethanol, resuspended in 50µl of DEPC treated water and stored at -75°C.

Assessment of all nucleic acids was performed by electrophoresis through 0.5% agarose gels to determine presence of either high molecular weight genomic DNA or good quality RNA assessed by the presence of 18 and 28S ribosomal bands. DNA and RNA samples were quantified by UV spectrophotometric analysis at 260 and 280 nm. An OD at 260nm of 1.0 is equivalent to an RNA concentration of 40µg/ml or a DNA concentration of 50µg/ml. The 260/280 ratio gives an indication of the sample purity with a ratio of greater than 1.8 indicating good nucleic acid purity.

2.2.4 Agarose gel electrophoresis

Materials Agarose, low melting point agarose, 1x TBE buffer, ethidium bromide (10 mg/ml), loading buffer (0.25% bromo-phenol blue [BPB], 40% sucrose).

Method Agarose gels (1.0 - 3.5%) were used to check PCR products for size and purity, while low melting point agarose gels were used to isolate PCR products for direct sequencing and mutation specific restriction enzyme digests. To the appropriate amount of agarose, 30ml of 1x TBE was added, heated in a microwave oven until dissolved and allowed to cool before the addition of 2µl of ethidium bromide. The gel was poured into a mould and allowed to set. Appropriate volumes of PCR products mixed with the loading buffer were loaded into the wells and electrophoresed in 1x TBE under appropriate current and voltage conditions. PCR products were visualised on a UV transilluminator. Polaroid photographic equipment was used to take a permanent record.

2.2.5 Reverse transcription (RT)

Materials 10x reaction buffer, $MgCl_2$ (25mM), deoxynucleotides (dNTPs, 10 mM each), RNAse inhibitor, AMV reverse transcriptase, oligo dT or random hexamers (all Promega or Bioline), water.

Method In a 20µl reaction volume, the final concentrations were 1x reaction buffer, 5.25 mM $MgCl_2$, 1 mM each dNTP, 20 units RNAse inhibitor, 3.75 units reverse transcriptase, 250ng oligo dT or random hexamers, water and 1µg RNA. Reaction mixtures were incubated at 42°C for 1 hour followed by 95°C for 5 minutes to denature the enzyme. The resulting cDNA was stable for several weeks when stored at 4°C.

2.2.6 Polymerase chain reaction (PCR)

Materials 10x reaction buffer, $MgCl_2$ (25mM), dNTPs (10mM each), Taq DNA polymerase (all Promega or Bioline), oligonucleotide primers, water

Method A mastermix of the reagents was made (minus the template) to ensure identical concentrations of each reagent in each reaction tube with final concentrations of 1x reaction buffer, 1.0-2.5 mM $MgCl_2$ optimised for maximal amplification, 1 mM each dNTP, 80 ng each of forward (5') and reverse (3') oligonucleotide primer an water to a final volume of 17µl or 18µl. Either cDNA (2µl) or DNA (1µl) was used as a template. A drop of mineral oil was added to each tube as an evaporation barrier. Where a hot-start method was employed, the tubes were placed in the thermocycler block and heated to 95°C for 5 minutes. The temperature was then lowered to 85°C and 1µl of Taq DNA polymerase (0.5 units) added to each tube prior to thermal cycling. Thermal cycling consisted of a denaturation step at 95°C, a primer annealing step where temperature was dependent on the composition of the primers, and an extension step at 72°C. The number of cycles varied between 25-35 depending on template abundance. A final extension was carried out at 72°C for 5 minutes.

2.2.7 Single strand conformation polymorphism analysis (SSCP)

Materials 10x TBE, non-denaturing polyacrylamide gel (6% polyacrylamide, crosslinker ratio 37.5:1), glycerol, TEMED, APS (10%), 3MM chromatography paper (Whatman), Hyperfilm-MP (Amersham), denaturing solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), α-[^{32}P]-dCTP (specific activity 6000 Ci/mmol, Amersham).

Method PCR was performed as described in Chapter 2.7 with the addition of 0.2µl ^{32}P-dCTP to each reaction. Radioactive PCR product (2µl) was added to a mixture consisting of 10µl 0.1% SDS/10 mM EDTA and 11µl denaturing solution. Samples were heated to 95°C for 5 minutes and then immediately placed on ice before loading 5µl on to the polyacrylamide gels. To maximise the probability of detecting mutations, 3 different electrophoretic conditions were used: (1) no glycerol, room temperature, 5 hours at 15W; (2) 10% glycerol, room temperature, 5 hours at 25W; and (3) no glycerol, 4°C, 5 hours at 20W. After electrophoresis gels were dried and exposed overnight at room temperature to Hyperfilm-MP.

2.2.8 End-labelling of primers

Materials Primer to be labelled, γ-[^{32}P]-ATP (6000 Ci/mmol, Amersham), DNA 5' End-Labeling System (Promega).

Method 10 pmol primer was incubated with 10 units of T4 polynucleotide kinase in the appropriate buffer and 3µl γ-[^{32}P]-ATP for 30 minutes at 37°C. The enzyme was then inactivated by heating to 95°C for 5 minutes.

2.2.9 Sequencing of PCR products

PCR products were purified using the Wizard Plus PCR™ columns (Promega) and subjected to sequencing by one of the following methods.

fmol™ DNA Sequencing System

Materials fmol™ DNA sequencing system kit (Promega).

Method The sequencing primer selected was end-labelled as described in Chapter 2.9. Cycle sequencing methods are based on the ability of a DNA polymerase to extend a primer, hybridised to the template, until a chain terminating nucleotide is incorporated. Each sequence determination is carried out as a set of four separate reactions, each of which contains all four dNTPs supplemented with a limited amount of a different dideoxyribonucleoside triphosphate (ddNTP). Because ddNTPs lack the 3' hydroxy group necessary for chain elongation, the growing oligonucleotide is terminated selectively at G, C, A or T, depending on the respective dideoxy analogue in the reaction. Incorporation of the radiolabel permits visualisation of the sequencing products by autoradiography. The samples were run on denaturing 6% acrylamide/0.5 xTBE/7M urea gels at 1500V for 3 hours. After electrophoresis gels were dried and exposed overnight at room temperature to Hyperfilm-MP.

ABI 310 analysis

Materials BigDye™ Terminator Cycle Sequencing Ready Reaction, 310 Genetic Analyser Kit (ABI Prism).

Method This kit utilises a set of fluorescently labelled dye terminators. Sequence terminating cycling was performed by addition of 10ng cleaned PCR product to a single reaction mix containing the dye terminators, a DNA polymerase, $MgCl_2$ and buffer. The products were subsequently precipitated with 2.0µl Na acetate (3M, pH 4.6) and 50µl ethanol (95%) and washed in 75% ethanol. The samples were then dried, resuspended in Template Suppression Reagent™, denatured at 95°C for 5 minutes and placed on ice prior to electrophoresis on the 310 Genetic Analyzer.

2.2.10 Cloning of PCR products

Materials TA Cloning kit (Invitrogen), L-Broth (LB medium, Bio 101 Inc.), Agar (Calbiochem), ampicillin (Gibco), X-Gal (Sigma), IPTG (Sigma).

Method PCR products were cloned using the TA cloning system which utilises the single deoxyadenosine overhang at the 3' end of PCR products. Fresh PCR products were ligated into a vector (optimal ratio of insert to vector=1:1) with deoxythymidine overhangs at the insertion site. This was performed by incubating the PCR product with vector and appropriate ligation buffer in a final volume of 20µl at 4°C overnight. The vector was transformed into competent bacteria by heatshock at 42°C for 45 seconds. Transformed bacteria were selected by β-galactosidase activity by plating on LB medium plates (For 1L: 1x LB medium, 15 g agar, 1ml 10mg/ml ampicillin) and incubating at 37°C overnight. Plates were pre-coated with 40µl IPTG (100mM) and 20µl X-Gal (50mg/ml) allowed to absorb for 30 minutes at 37°C prior to use. Transformed clones (white colonies) were picked and expanded in LB medium. PCR was performed directly on the bacteria to select clones with the relevant mutation. PCR-SSCP (Chapter 2.8) was performed to verify the selection of clones containing the abnormality as compared to wild type and heterozygous patterns. PCR was performed on the mutant clones and products were sequenced as described in Chapter 2.10.

2.2.11 Mutation specific enzyme digests

Materials Restriction enzymes, restriction enzyme buffer, water.

Method Ten µl of PCR product was digested overnight at 37°C or 55°C with 10 units of the restriction enzyme in the appropriate buffer in a final volume of 20µl.

Samples were then electrophoresed through 2.5 or 3.5% agarose gels and bands visualised by ethidium bromide staining.

Chapter 3

RT-PCR detection of *CBFB-MYH11* in AML

3.1 Introduction

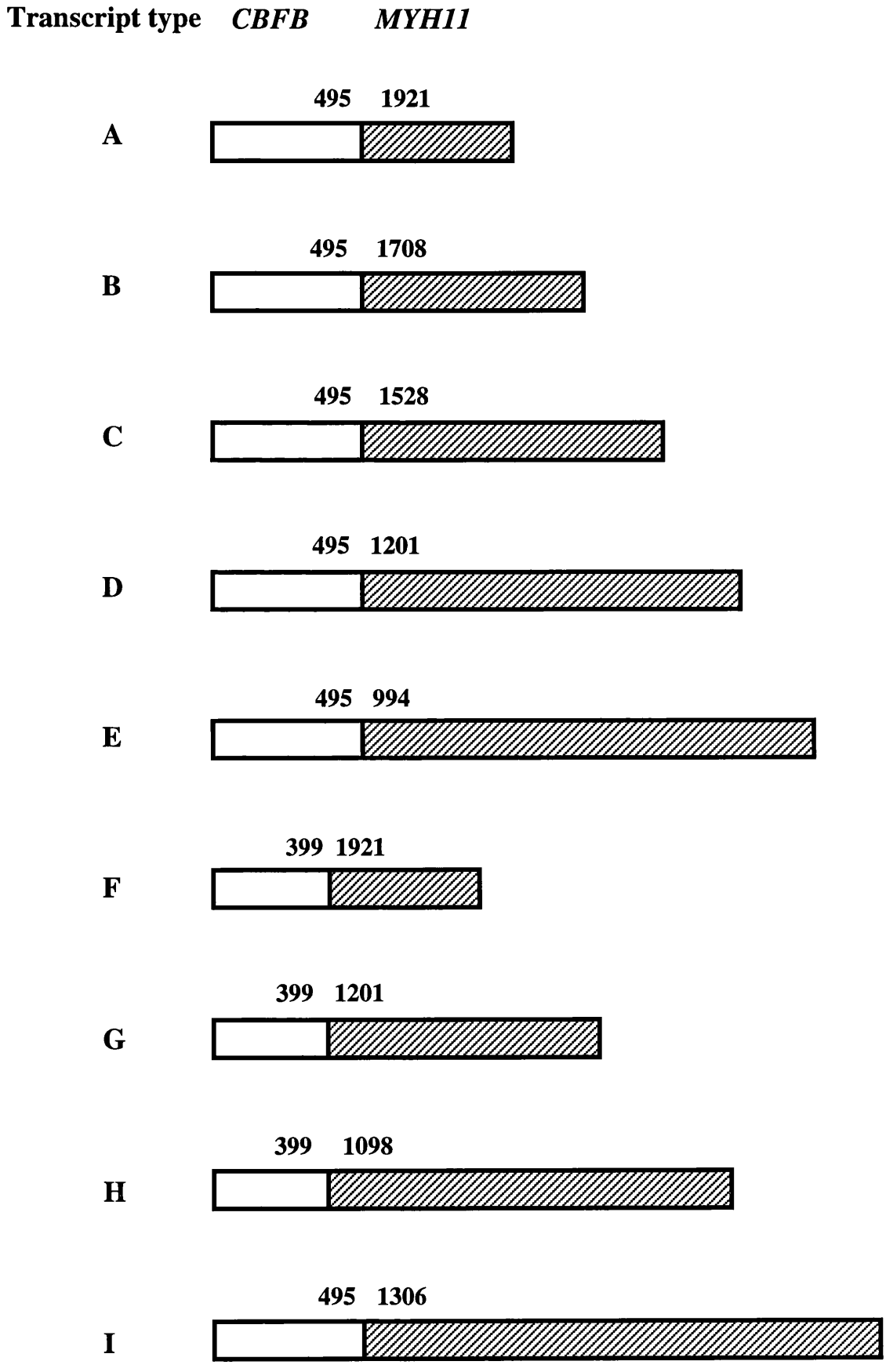
The pericentric inversion of chromosome 16, inv(16)(p13q22), and the balanced, less frequent translocation between the short and long arms of the same chromosome, t(16;16)(p13;q22), were first identified as consistent cytogenetic abnormalities in AML as partial deletions of the long arm of chromosome 16 (Arthur et al, 1983) and then as distinct inversions and translocations (Le Beau et al, 1983). These abnormalities were associated with acute myelomonocytic leukaemia with specific morphological features, most distinctly, the presence and variably increased numbers of dysplastic, immature eosinophils in the bone marrow and also distinct cytochemical properties of the blast cells (Bitter *et al*, 1984). AML with this typical morphology was named the M4Eo subtype according to FAB classification (Bennett *et al*, 1985). The abnormal eosinophils have been shown to be part of the leukaemic population and carry the inv(16) (Haferlach *et al*, 1996). The visible alteration in cytogenetic morphology brought about by the inv(16)(p13q22) is subtle and therefore readily overlooked in suboptimal metaphase preparations. The inv(16) is now known to be one of the most commonly observed cytogenetic abnormalities in *de novo* AML, seen in approximately 8-10% of all new cases (Walker *et al*, 1994; Liu *et al*, 1995). Although the inv(16) is most commonly associated with the FAB type M4Eo, this inversion has also been reported in other FAB types of *de novo* AML (Poirel *et al*, 1995; Monahan *et al*, 1996), in myelodysplastic syndromes (MDS) (Narayanan *et al*, 1993), in the myeloid blast crisis of chronic myeloid leukaemia (CML) (Enright *et al*, 1992) and occasionally in therapy related AML (Seymour *et al*, 1999).

The inversion consistently disrupts a myosin or smooth muscle heavy chain gene, *MYH11*, at 16p13 and the *CBFB* gene at 16q22 (Liu *et al*, 1993), which normally encodes

a subunit of the CBF transcription factor complex. An in-frame fusion messenger RNA was demonstrated that fused the first 165 amino acids of *CBFB* to three different breakpoints within the coding region of *MYH11*. Interruption of the *MYH11* locus was also reported in a larger group of patients with an inv(16) or t(16;16) (van der Reijden *et al*, 1993; Dauwerse *et al*, 1993). Further studies on larger numbers of patients with either an inv(16) or a t(16;16) have revealed that there is considerable heterogeneity in the breakpoint region of both genes involved in this abnormality with the predominant form being of type A (Liu *et al*, 1995; van der Reijden *et al*, 1995; Shurtleff *et al*, 1995; Novak *et al*, 1995; Costello *et al*, 1997; Springall *et al*, 1998; Viswanatha *et al*, 1998) (Figure 3.1).

Because of the prognostic implications of those AML patients with an inv(16) (see Chapter 1) it has become increasingly important to detect this abnormality as it will influence the treatment strategy employed. Cytogenetic analysis is essential not only to detect the inv(16) but also to determine if there are any other numerical or structural abnormalities which may influence the prognosis. Common numerical cytogenetic abnormalities often observed in association with inv(16) are trisomy 22 (Litmanovich *et al*, 2000) and, less commonly, the pan myeloid malignancy aberration trisomy 8 (Johansson *et al*, 1994). These secondary abnormalities have no effect on the overall prognosis of patients with inv(16) (Grimwade *et al*, 1998). Detection of the rearrangements involved in the inv(16) may also be achieved by the use of fluorescence *in situ* hybridisation (FISH) (Dauwerse *et al*, 1999). This is of value in the resolution of complex karyotypes involving the *CBFB* and *MYH11* genes (Dierlamm *et al*, 1998; Martinez-Climent *et al*, 1999). FISH has also shown that there is deletion of a segment of the p arm in a proportion of patients with inv(16) (Dauwerse *et al*, 1993). This deletion was useful in confirming that *CBFB-MYH11*, rather than *MYH11-CBFB*, is the critical

Figure 3.1 Summary of *CBFB-MYH11* fusion transcripts detected by RT-PCR - numbers indicate position of breakpoint nucleotide of the appropriate gene.



fusion product for inv(16) leukaemogenesis (Marlton *et al*, 1995). It has been postulated that the good prognosis of inv(16) AML was due to this deleted region as it contains the gene for the multi-drug resistance associated protein (Kuss *et al*, 1994), but further studies on larger numbers of patients did not find any appreciable differences in outcome between inv(16)/t(16;16) patients with and without this deletion (Martinet *et al*, 1997; Dohner *et al*, 2000).

At the molecular level rearrangements of the *MYH11* gene observed in the formation of the inv(16) can be detected by Southern blotting using a genomic probe against the breakpoint region of the fusion gene (van der Reijden *et al*, 1996) This technique though, may be time consuming. As inv(16) creates a transcriptionally active fusion gene it is possible to detect *CBFB-MYH11* fusion transcripts using sensitive RT-PCR assays (Hebert *et al*, 1994). A number of studies have shown that *CBFB-MYH11* mRNA can be found in all cases of karyotypically detectable inv(16) or t(16;16) (Claxton *et al*, 1994; Tobal *et al*, 1995; van der Reijden *et al*, 1995) but not in all cases of morphological M4Eo (Poirel *et al*, 1995). It has also been shown by screening RNA from larger populations of AML patients that the specific fusion transcripts can be occasionally detected where there is no cytogenetic evidence of the associated abnormality (Poirel *et al*, 1995). The full length protein encoded by *CBFB-MYH11* has been identified (Claxton *et al*, 1996; Liu *et al*, 1996). This has allowed the generation of antibodies to the protein which may be developed for diagnostic and minimal residual disease detection (Viswanatha *et al*, 1998).

As visualisation of inv(16) may be difficult if metaphase preparations are of a poor quality it is likely that some cases are undetected at diagnosis and assigned to the incorrect prognostic category. This is particularly likely to occur if the bone marrow

morphology is not that associated with M4Eo. In this chapter results are presented on the assessment of a nested RT-PCR technique for the detection of *CBFB-MYH11* transcripts and compared with results using conventional cytogenetics.

3.2 Materials and methods

3.2.1 Patients

Three hundred and twenty one patients with AML entered into the MRC AML 10, 11 and 12 trials were studied at diagnosis. All patients studied were 16 years of age or over. Morphological FAB type was based on the referring clinicians' diagnosis and by central review independently of cytogenetic results. Samples were analysed from cases of M1 (n=56), M2 (n=75), M3 (n=55), M4/M4Eo (n=103) and M5 (n=32). There was a bias in the number of AML M4 samples studied, with 32% of cases being of this subtype compared with the overall incidence in the UK MRC AML 10 trial of 20% (Hann *et al.*, 1997).

3.2.2 Cytogenetics

All patients had karyotypic analysis which was performed either in local UK cytogenetic laboratories or, where no such service was available, at the MRC AML trials cytogenetic centre at University College Hospital, London co-ordinated by Mrs Helen Walker. Both bone marrow and peripheral blood specimens were cultured according to standard methods. Twenty or more cells were analysed to detect clonal abnormalities. These were defined by the presence of 2 metaphase cells with identical structural or numerical abnormalities and reported in accordance with ISCN guidelines (Mitelman, 1995).

3.2.3 Sample preparation

Samples were received by post from participating centres within 4 days of being taken. Peripheral blood or bone marrow samples were centrifuged at 3500rpm for 5 minutes. The nucleated cells formed a buffy coat layer which was aspirated and lysed in GTC-ME. RNA was extracted using phenol/chloroform as described in Chapter 2. RNA was extracted from at least $1x10^7$ mononuclear cells and RNA quality was determined by visualisation of clear ribosomal RNA bands on agarose gel electrophoresis with minimal DNA contamination.

3.2.4 RT-PCR analysis

Total cellular RNA (1µg) was used in a 20µl reverse transcription reaction (see Chapter 2). Ten µl of the reverse transcription reaction was used in a 50µl PCR with a final concentration of 1 x PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 units of Taq DNA polymerase and 200 ng each of primers 1 and 2M (Claxton *et al*, 1994) (Table 3.1) which amplify a PCR product of 414bp in length for the most common type of transcript (Poirel *et al*, 1995). After 95°C for 3 minutes, 10 cycles were performed consisting of 95°C (1 minute), 60°C (1 minute) and 72°C (1 minute) followed by 25 cycles where the annealing temperature was reduced to 56°C (1 minute). This was then followed by a final extension time at 72°C (5 minutes). A 50µl nested PCR was performed using 1µl of the first round product in a mix containing a final concentration of 2.5mM $MgCl_2$ and 200 ng each of primers 3 (Claxton *et al*, 1994) and 5M (Tobal *et al*, 1995) for 30 cycles. This gives a product of 209bp for the most common type of *CBFB-MYH11* transcript. All PCR experiments were performed twice using the same cDNA and no RNA or RNA from HL60 cells were used as negative and positive controls respectively. RNA integrity was

Table 3.1 PCR primers used for the detection of *CBFB-MYH11* and *ABL* transcripts.

Primer	*Sequence* (5'→3')
1	CAGGCAAGGTATATTTGAAGG
2M	CTCCTCTTCTCCTCATTCTGCTC
3	GTCTGTGTTATCTGGAAAGGCTG
5M	CCCGCTTGGACTTCTCCAGC
S1	TTAGCACAACAGGCCTTTGAA
ABL I	GCTTCACACCATTCCCCATT
ABL D	TTCAGCGGCCAGTAGCATCTGACTT

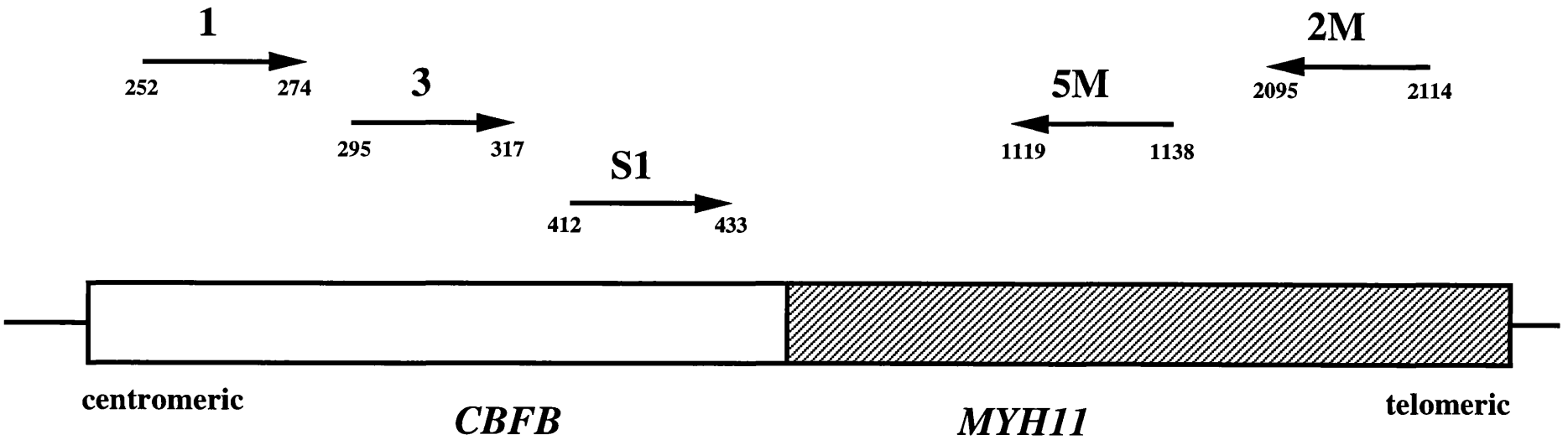


Figure 3.2 Schematic diagram of *CBFB-MYH11* fusion mRNA showing primer positions.

also assessed by the successful amplification of normal *ABL* transcripts. ABL was selected as a an RT-PCR control as it is expressed at approximately the same level as *CBFB-MYH11* (Lion, 1996). Two µl of the reverse transcription reaction was used in a 50 µl PCR with a final concentration of 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.5 units of Taq DNA polymerase and 200 ng of each primers I and D (Hernandez *et al*, 1990) (Table 3.1). After 5 minutes at 95°C the reaction 30 cycles were performed consisting of 95°C (1 minute), 60°C (1 minute) and 72°C (1 minute) followed by a final extension at 72°C (5 minutes). This gives a normal *ABL* PCR product of 219 bp.

3.2.5 Sensitivity

In order to determine the lower limits of reproducible transcript detection, mononuclear cells from a known case of AML M4Eo at diagnosis with an inv(16) were serially diluted in normal peripheral blood mononuclear cells. RNA was extracted from mixtures containing one inv(16) cell in 10, 10^2, 10^3, 10^4, 10^5 or 10^6 *CBFB-MYH11* negative mononuclear cells and the RT-PCR assay performed as described above with 1µg of RNA.

3.2.6 Sequencing

Specificity of PCR products was confirmed by 2 methods of sequencing. (1) PCR products were electrophoresed through low melting point agarose, the required band excised, cleaned and sequenced using an end-labelled primer S1 (Table 3.1) derived from the *CBFB* portion of the fusion transcript (Shurtleff *et al*, 1995) (see Chapter 2) or (2) PCR products were cleaned and subjected to automated sequencing on an ABI 310

Genetic Analyser using primers 3 and 5M with the Big DYE terminator reaction kit (both Applied Biosystems) (see Chapter 2).

3.2.7 Restriction enzyme digest

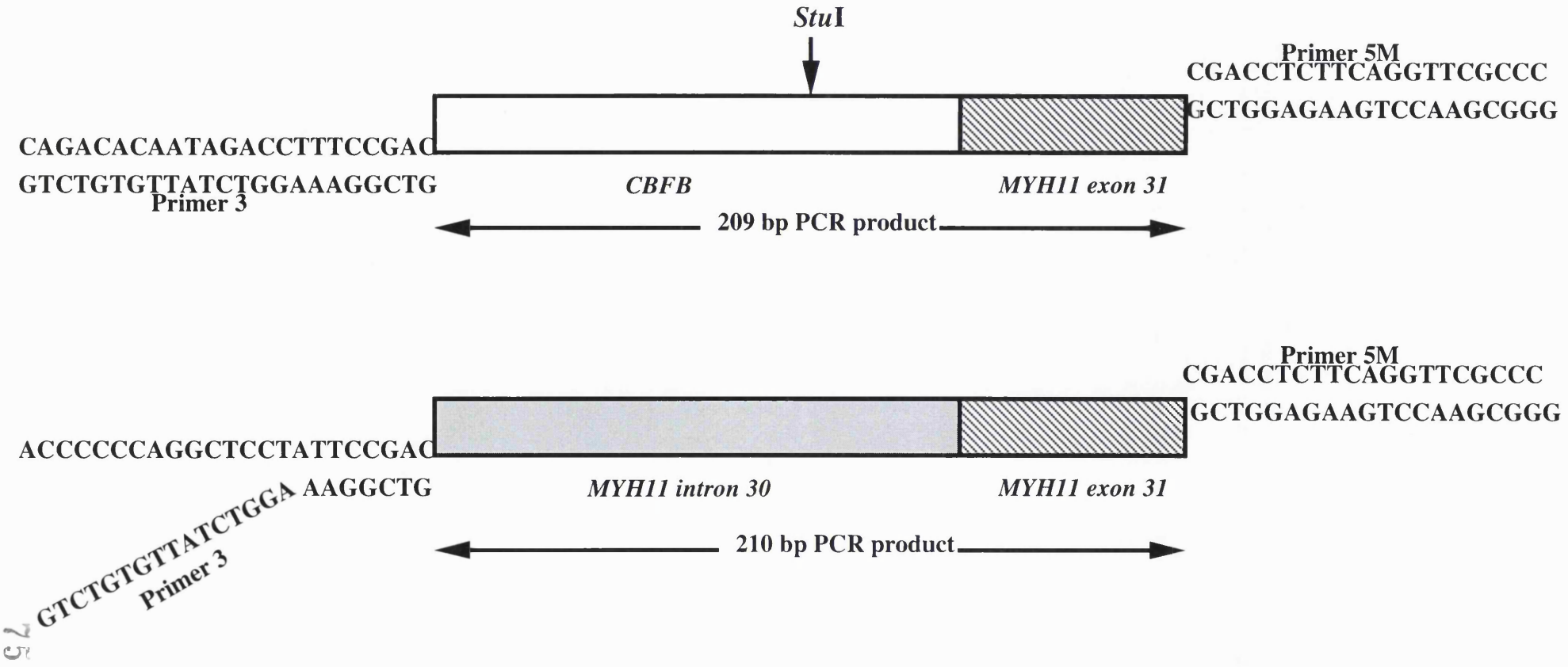
A high proportion of false positives has been demonstrated in patients without inv(16) using the primer sets used in this study (Hackwell *et al*, 1999). This is due to the 5' nested *CBFB* primer cross-hybridising with an intronic sequence of contaminating *MYH11* genomic DNA, which results in the amplification of a 210bp sequence from DNA of similar size to the expected 209bp *CBFB-MYH11* RT-PCR product (Figure 3.3). In order to further confirm the specificity of the technique nested RT-PCR was performed with RNA from 5 patients with inv(16), 10 cases of cytogentically negative inv(16) but previously shown to be *CBFB-MYH11* RT-PCR positive, 10 haematologically normal controls (HNCs) and nested PCR with DNA of 10 HNCs. PCR products were subjected to digestion with the enzyme *Stu*I (Chapter 2). The correct *CBFB-MYH11* fusion product contains one cutting site giving digestion products of 100bp and 109bp. The product arising from contaminating DNA has no *Stu*I cutting sites.

3.3 Results

3.3.1 Sensitivity

Using nested RT-PCR it was found that this technique could reliably detect one leukaemic cell expressing *CBFB-MYH11* fusion transcripts in 10^5 peripheral blood mononuclear cells (Figure 3.4).

Figure 3.3 Composition of the specific 209 bp *CBFB-MYH11* RT-PCR product and the 210 bp PCR product. Homology exists between the 7 nucleotides of *MYH11* intron 30 and the 3' end of primer 3, which with primer 5M, results in the amplification of the 210 bp anomalous sequence.



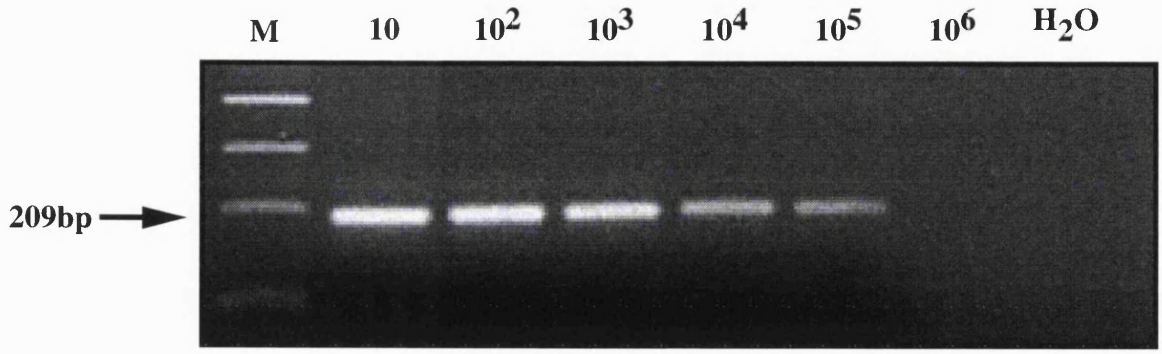


Figure 3.4 Sensitivity of *CBFB-MYH11* RT-PCR. Number of MNC per inv(16) cell. M: size markers.

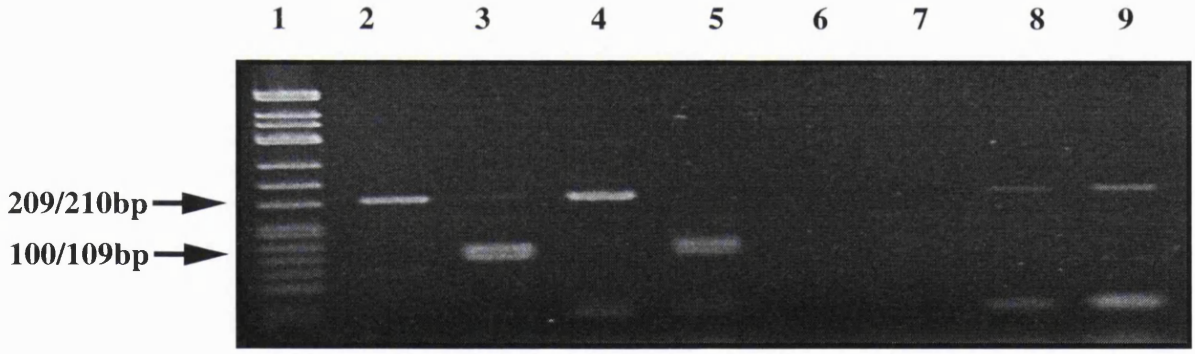


Figure 3.5 *Stu*I restriction enzyme digestion of *CBFB-MYH11* RT-PCR products. Lane 1: DNA size markers; Lane 2: inv(16) patient; Lane 3: *Stu*I digest of Lane 2; Lane 4: cryptic CBFβ-MYH11 patient; Lane 5: *Stu*I digest of Lane 4; Lane 6: normal bone marrow; Lane 7: *Stu*I digest of Lane 6; Lane 8: DNA from non AML patient; Lane 9: *Stu*I digest of Lane 8.

3.3.2 Incidence of inv(16) by cytogenetics

Of the 321 AML patients studied by conventional cytogenetics, 21 (6.5%) were found to have an inv(16) (Table 3.2). This series included 17 cases with morphological M4Eo and 4 cases with the M2 subtype.

3.3.3 Incidence of *CBFB-MYH11* fusion transcripts

All 321 patients had detectable normal ABL transcripts indicating integrity of the RNA used. Thirty three of the 321 cases (10.3%) were RT-PCR positive for *CBFB-MYH11* fusion gene transcripts. All 21 cases that had an inv(16) by conventional cytogenetics were also positive by RT-PCR which included the 17 cases of M4Eo. Twelve cases were PCR positive (M1, n=5; M4, n=5; M5, n=2) but no abnormality of chromosome 16 had been reported on karyotype analysis (Figure 3.1). Review of the metaphases from these cases showed that the quality was inadequate in 3 cases, and in the remaining 9 the preparations were of adequate quality to confirm the absence of gross chromosome 16 abnormalities.

3.3.4 Specificity of RT-PCR products

To confirm the specificity of the PCR products, the nested PCR product from 14 cases was sequenced across the molecular fusion. Three of these patients had cytogenetic evidence of an inv(16) and 11 did not, including the 9 patients with good quality metaphases and no evidence of chromosome 16 abnormalities. In each case the sequence confirmed the presence of the most common *CBFB-MYH11* fusion gene, type A. Restriction enzyme digest with *Stu*I further confirmed the product in 5 cases with inv(16)

Table 3.2 Results of cytogenetic and molecular screening.

FAB type	Number studied	Inv(16) detected by cytogenetics	*CBFB-MYH11* detected by RT-PCR	*CBFB-MYH11* but not inv(16)	Frequency in AML*	Observed frequency of CBFB-MYH11**	Calculated frequency of *CBFB-MYH11*
M0	-	-	-	-	<5%	-	-
M1	56	0 (0%)	5	5	20%	9%	1.8%
M2	75	4 (5%)	4	0	30%	5%	1.5%
M3	55	0 (0%)	0	0	10%	0%	0%
M4	86	0 (0%)	5	5	20%	6%	1.2%
M4Eo	17	17 (100%)	17	0	5%	100%	5.0%
M5	32	0 (0%)	2	2	10%	6%	0.6%
M6	-	-	-	-	<5%	-	-
M7	-	-	-	-	<5%	-	-
Total	321	21 (6.5%)	33 (10.3%)	12 (3.7%)			10.1%

* Frequencies based on data from Heim & Mitelman (1995)

** Data from this study

and 10 cases with molecular evidence only (Figure 3.5). PCR products were not observed using RNA from HNCs. No digestion was observed in the nested PCR products of genomic DNA from HNCs indicating that these represent amplification of a *MYH11* sequence due to cross-hybridisation of primer 3.

3.4 Discussion

Analysis of large numbers of patients diagnosed with AML has identified a number of critical prognostic indicators. Of these, the presence of specific cytogenetic abnormalities at diagnosis are known to have a significant effect on overall survival (Keating *et al*, 1988; Fenaux *et al*, 1989; Dastugue *et al*, 1995). Patients with the inv(16) have been shown to have relatively better survival rates (Larson *et al*, 1986; Plantier *et al*, 1994) and in the present study 10.3% (33/321) of all AML patients had molecular evidence of the *CBFB-MYH11* fusion. Of these, over one third (12/33) had not been detected by conventional cytogenetic techniques. In some cases this was due to poor quality metaphase preparations, but in most cases the inversion was not detectable at the gross karyotypic level. All cases of M4Eo were positive for inv(16) at both the cytogenetic and molecular level. The samples analysed in this study represent some skewing to the M4 FAB subtype as samples from these patients appear to have been preferentially sent for central DNA/RNA banking. To calculate the proportion of AML cases that have a *CBFB-MYH11* fusion, the observed frequencies have been adjusted for the incidence of each subtype based on the data of Heim & Mitelman (1995) (Table 3.2). Overall, 10.1% of all AML cases would have a *CBFB-MYH11* fusion. The adjusted data (Table 3.2) suggest that M4Eo accounts for only one half (17/33) of cases with *CBFB-MYH11* transcripts detected by RT-PCR. The failure to detect this abnormality was restricted to those cases not deemed to have classical M4Eo morphology as *CBFB-MYH11* transcripts were

detected in cases of M1, M4 and M5. Inv(16)–/*CBFB-MYH11*+ AML patients may have significant dysplastic eosinophilic and/or monocytic components and may represent cases of true AML M4Eo without a cytogenetically detectable inv(16).

Other studies have since verified the existence of patients with detectable *CBFB-MYH11* fusion transcripts but no cytogenetic evidence inv(16) (Costello *et al*, 1997; Ritter *et al*, 1997; Wong & Kwong, 1999; Mitterbauer *et al*, 2000). This is thought, and has been demonstrated in one case (Aventin *et al*, 2000), to be due to either insertion of part of the *MYH11* gene into the *CBFB* gene or vice versa. This type of phenomenon has been described in rare cases of acute promyelocytic leukaemia (APL) whereby interstitial insertion occurs of either the 15q derived *PML* gene into the 17q *RARA* locus or vice versa, creating a transcriptionally active fusion gene (Hiorns *et al*, 1994; Lafage-Pochitaloff *et al*, 1995; Grimwade *et al*, 1997). In a recent study (Pirc-Danoewinata *et al*, 2000) an AML patient has been described with evidence of a *CBFB-MYH11* fusion transcript but no inv(16) by conventional cytogenetics or by FISH analysis with whole chromosome painting. However, application of locus-specific probes confirmed the presence of a masked inv(16) indicating that the events leading to creation of this abnormality were complex and involved deletions of both the long and short arms of chromosome 16. In general it is thought that the most likely explanation for formation of the *CBFB-MYH11* fusion is an insertion of part of the *MYH11* into the *CBFB* gene (Aventin *et al*, 2000), or possibly a double inversion which would require even more chromosomal breaks. It has also been recently shown that another abnormality involving a larger, inter-arm insertion of 16p13 can also give rise to a transcriptionally active *CBFB-MYH11* fusion gene (O'Reilly *et al*, 2000).

A sequence similarity between the 7bp at the 3' end of primer 3 and a sequence within intron 30 of the *MYH11* gene has been shown to exist (Hackwell *et al*, 1999). In theory this could result in the amplification of a 210bp product from contaminating genomic DNA which would be indistinguishable from the 209bp *CBFB-MYH11* RT-PCR product. In 11 cases of cryptic inv(16) where RNA was available for study this was not the case. Possible explanations for this would be DNA contamination of RNA samples, inadequate stringency of annealing temperature or use of an excessive number of amplification cycles. Excessive DNA contamination of RNA samples would be the most likely candidate in the study by Hackwell *et al*, (1999) as it is practically very difficult to obtain DNA free RNA in the laboratory, even with Caesium chloride or phenol/chloroform extraction techniques. Higher purity RNA may be obtained with the use of DNAse enzymes. However, sequencing and restriction enzyme digestion of 10 cases confirmed that this was not a problem in the present studies.

Of the 33 patients with molecular evidence of *CBFB-MYH11* transcripts, 30 (91%) achieved a complete remission. This included 20/21 patients with an inv(16) (95%) and 10/12 (83%) who only had molecular evidence of the fusion. This difference is not significant. It should also be noted that 7/12 patients with negative cytogenetic findings were over the age of 60 years compared to 3/21 of those with positive cytogenetics thus highlighting the different mechanisms of AML pathogenesis in younger and older patients. If the presence of *CBFB-MYH11* fusion transcripts has the same prognostic significance as cytogenetically detectable inv(16) then the older patients with molecular evidence of inv(16) alone may benefit from more intensive treatment protocols.

Some controversy exists as to whether the detection of residual disease in remission is consistent with a relatively good outcome (Costello *et al*, 1997a; Marcucci *et al*, 1997;

Martinelli *et al*, 2000). Quantitative evaluation of residual disease has been made possible with the use of competitive (Evans *et al*, 1997; Laczika *et al*, 1998) and 'real-time' (Marcucci *et al*, 2001) RT-PCR strategies. FISH probes for the detection of inv(16) have only recently become available and as yet residual disease detection by this method has not been evaluated thoroughly (Mancini *et al*, 2000).

This study suggests that if the presence of the inv(16) in AML is to be used to direct stratification of treatment, then it should be considered necessary to screen all new cases of AML for the presence of *CBFB-MYH11* fusion transcripts to increase detection of patients with apparently good-risk disease. Correct detection of the *CBFB-MYH11* fusion gene would also allow identification of those patients who might be eligible for future therapies targetting the resulting CBFB-MYH11 protein.

Chapter 4

RT-PCR detection of *AML1-ETO* in AML

4.1 Introduction

The balanced translocation between the long arms of chromosomes 8 and 21, t(8;21)(q22;q22) is one of the commonest translocations observed in AML, particularly in children and younger adults. Recent reports suggest that this abnormality is seen in 8-15% of all AML patients less than 60 years of age (van der Reijden *et al*, 1997). This abnormality is seen most often in patients with FAB type M2 but has also been described in numerous cases with M0, M1, M4 and M5. This translocation and several three way variant translocations involving chromosomes 2, 6, 15, 19 an 20 (Gallego *et al*, 1994; Hagemeijer *et al*, 1984; Wong *et al*, 1998) result in the creation of a fusion gene comprised of the 5' end of the *AML1* gene from 21q22 fused to the 5' end of the *ETO* gene from chromosome 8q22. *AML1* encodes the DNA binding subunit of the AML1-CBFB transcription factor complex which controls a number of genes necessary for normal haemopoiesis (see Chapter 1).

AML patients with t(8;21) at diagnosis are deemed to have a relatively good overall survival (Fenaux *et al*, 1990; Bloomfield *et al*, 1998). It has therefore become increasingly important to detect this translocation as this will influence treatment: current MRC protocols defer the use of bone marrow transplantation in first remission for these patients as there is no benefit compared with intensive chemotherapy (Burnett *et al*, 1998). Cytogenetic analysis is essential not only to detect the t(8;21) but also to detect any other structural or numerical abnormalities which may influence the prognosis. Among other secondary abnormalities reported in association with t(8;21), the most common are loss of a sex chromosome or variable deletions of chromosome 9q (Rege *et al*, 2000). One report suggested that abnormalities of 9q in t(8;21) AML are associated with a poor prognosis (Schoch *et al*, 1996), but studies with larger numbers of patients

treated with equivalent therapy have shown that this additional abnormality does not alter the relatively good prognosis of t(8;21) patients (Grimwade *et al*, 1998; Byrd *et al*, 1999). t(8;21) AML is also associated with extramedullary leukaemia and granulocytic sarcomas (Swirsky *et al*, 1984). Presence of extramedullary AML in t(8;21) patients is associated with a significantly worse overall survival than in those t(8;21) AML patients without this feature (Byrd *et al*, 1997). Another marker which may be of value in the further stratification of t(8;21) AML is CD56 expression on the leukaemic blasts which has been shown in one study to confer a worse prognosis (Baer *et al*, 1997).

The t(8;21) breakpoints on chromosome 21 were found to be clustered within a limited region of the *AML1* gene (Miyoshi *et al*, 1991). *AML1* spans 260 kb of DNA and its expression has been shown to be controlled by 2 promoter regions (Miyoshi *et al*, 1995; Levanon *et al*, 2001). The gene consists of 9 exons which encode 2 distinctive regions of the protein: exons 3-5 encode the evolutionarily conserved runt domain (Daga *et al*, 1992; Erickson *et al*, 1992) while 3' exons encode the C terminal transactivating domain. t(8;21) breakpoints were subsequently narrowed to a single intron, intron 6 (Shimizu et al, 1992; Tighe *et al*, 1993) which is 3' of the runt domain. The 5' end of *AML1* is fused to almost the complete *ETO* gene (also named *MTG8* or *CDR*) (Nisson *et al*, 1992; Miyoshi *et al*, 1993). The *ETO* gene, at chromosome 8q22, consists of 13 exons distributed over 87 kb of genomic DNA (Wolford & Prochazka, 1998). This gene encodes a regulator of transcription (Erickson *et al*, 1994) that is capable of recruiting proteins into a repression complex (Lutterbach *et al*, 1998; Gelmetti *et al*, 1998). As a consequence of the t(8;21), *AML1* sequences 3' of the runt domain are replaced by the majority of the coding region of *ETO*. The resultant AML1-ETO protein directly represses AML1-mediated transcriptional activation.

As the breakpoints in both genes on the der(8) chromosome are constant (Tighe *et al*, 1993), the fusion gene or its transcript are detectable using a variety of techniques. Southern blotting has been used to reliably confirm the presence of a t(8;21) at the molecular level in all patients studied using probes derived from breakpoint regions of *AML1* (Maseki *et al*, 1993; Zhang *et al*, 1994) and *ETO* (Nucifora *et al*, 1993a). As the junction of the fusion between *AML1* and *ETO* is constant in all cases of t(8;21) it is possible to detect this abnormality at the molecular level using RT-PCR techniques (Downing *et al*, 1993; Kozu *et al*, 1993). Detection of the *AML1-ETO* fusion may also be achieved by FISH methods (Sacchi *et al*, 1995; Paskulin *et al*, 1998). The latter has proven useful in cases where no RNA is available for RT-PCR (Hagemeijer *et al*, 1998) or in the resolution of variant translocations involving *AML1* and *ETO* which may be hidden within an unusual or complex karyotype (Harrison *et al*, 1999).

As RT-PCR has a high sensitivity, i.e. with 2 rounds of PCR it is possible to detect one leukaemic cell in 10^5-10^6 non-leukaemic cells (van Dongen *et al*, 1999), and is relatively rapid to perform, this methodology has been adopted for the detection of minimal residual disease in t(8;21) AML. Several studies have suggested that *AML1-ETO* transcripts are either absent or below the level of detection in patients in long term remission (Satake *et al*, 1995; Sakata *et al*, 1997). It has also been suggested that the rate at which patients become RT-PCR negative after sequential courses of chemotherapy is correlated with overall survival, i.e. the sooner RT-PCR negativity is achieved the more favourable the outcome (Morschauser *et al*, 2000). By increasing the RT-PCR sensitivity using nested primers in a second round of PCR, the present consensus reached is that it is possible to detect the persistence of the fusion gene in the bone marrow of all patients in long-term remission after chemotherapy (Chang *et al*, 1993; Kusec *et al*, 1994; Nucifora

et al, 1993b; Saunders *et al*, 1994), auto- or allo-BMT (Jurlander *et al*, 1996; Miyamoto *et al*, 1996; Elmaagacli *et al*, 1997).

As the presence of a t(8;21) at presentation of AML is highly indicative of a relatively favourable response to chemotherapy and persistence of long-term remission, it is highly desirable to identify all patients with this abnormality, both at the cytogenetic and molecular level, to determine optimal treatment. It is also a possibility that this translocation may lie undetected at the cytogenetic level due to poor metaphase quality, analysis of non-malignant clones or on the assumption that t(8;21) is invariably associated with M2 morphology. In this chapter results are presented on the assessment of a nested RT-PCR technique for the detection of *AML1-ETO* transcripts compared with results using conventional cytogenetics.

4.2 Materials and methods

4.2.1 Patients

Three hundred and ninety six patients with AML entered into the MRC AML 10, 11 and 12 trials were studied at diagnosis. The age of the patients ranged from 17-70 years, median 48. Morphological FAB type was based on the referring clinicians' diagnosis and on central review independent of cytogenetic results. Samples were analysed from cases of M0 (n=5), M1 (n=85), M2 (n=131), M3 (n=49), M4 (n=79), M5 (n=35), M6 (n=8) and M7 (n=4). There was a bias in the number of AML M1, M2 and M4 samples studied, with 75% of cases being of these subtypes as compared with the overall incidence in the UK MRC AML 10 trial of 64% (Hann *et al*, 1997).

4.2.2 Cytogenetics

All patients had karyotypic analysis which was performed either in local UK cytogenetic laboratories or, where no such service was available, at the MRC AML trials cytogenetic centre at University College Hospital, London co-ordinated by Mrs Helen Walker. Both bone marrow and peripheral blood specimens were cultured according to standard methods. Twenty or more cells were analysed to detect clonal abnormalities. These were defined by the presence of 2 metaphase cells with identical structural or numerical abnormalities and reported in accordance with ISCN guidelines (Mitelman, 1995).

4.2.3 Sample preparation

Samples were received by post from participating centres within 4 days of being taken. Peripheral blood or bone marrow samples were centrifuged at 3500rpm for 5 minutes. The nucleated cells formed a buffy coat layer which was aspirated and lysed in GTC-ME. RNA was extracted as described in Chapter 2 from at least $1x10^7$ mononuclear cells (MNC) and RNA quality was determined by visualisation of clear ribosomal RNA bands on agarose gel electrophoresis with minimal DNA contamination.

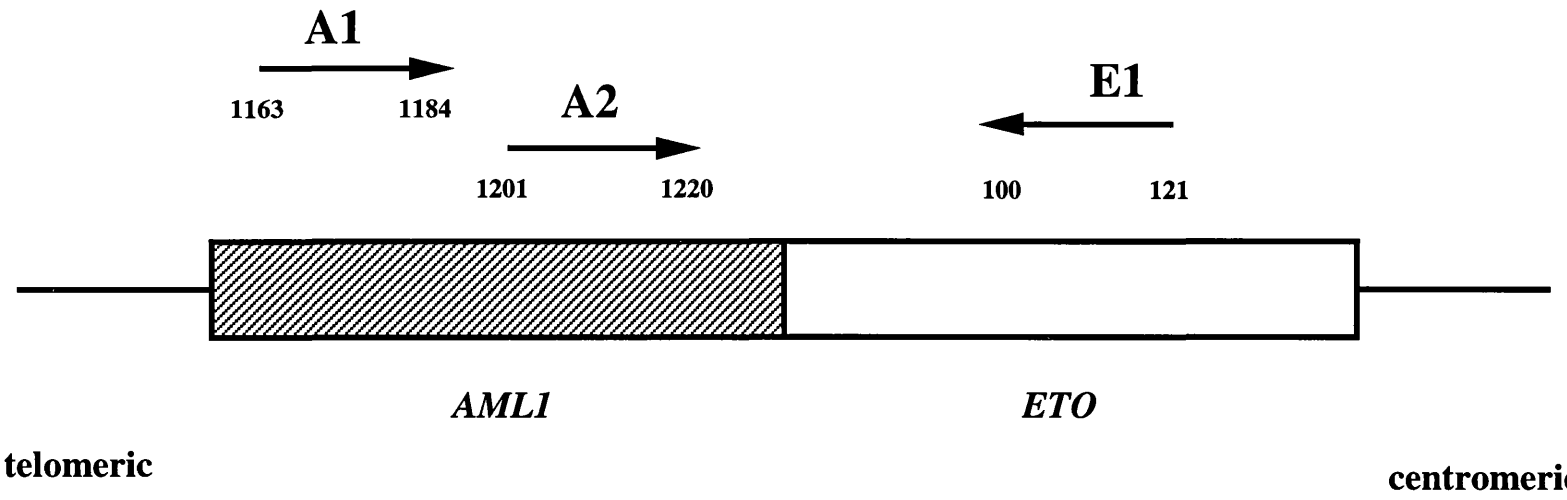
4.2.4 RT-PCR analysis

Total cellular RNA (1µg) was used in a 20 µl reverse transcription reaction (see Chapter 2). Ten µl of the reverse transcription reaction was used in a 50µl PCR with a final concentration of 1 x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 units of Taq DNA polymerase and 200 ng each of primers A1 (Miyoshi *et al*, 1991) and E1 (Chang *et al*, 1993) (Table 4.1) which amplify a PCR product of 261bp in length (Figure 4.1). After

Table 4.1 PCR primers used for the detection of *AML1-ETO* transcripts.

Primer	Sequence (5'→3')
A1	ATGACCTCAGGTTTGTCGGTCG
A2	AGCTTCACTCTGACCATCAC
E1	TGAACTGGTTCTTGGAGCTCCT

Figure 4.1 Schematic diagram of *AML1-ETO* fusion mRNA showing primer positions on the der(8) chromosome. A1 and A2 numbered according to GenBank entry D90525 and E2 numbered according to Erickson *et al* (1993).



95°C for 5 minutes, 35 cycles were performed consisting of 95°C (1 minute), 58°C (1 minute) and 72°C (1 minute) followed by a final extension time at 72°C (5 minutes). A 50 µl semi-nested PCR was performed using 1µl of the first round product in a mix containing a final concentration of 1 x PCR buffer, 1.5mM $MgCl_2$, 0.2 mM dNTPs, 0.5 units Taq DNA polymerase and 200 ng each of primers E1 and A2 (Miyoshi *et al*, 1991) for 30 cycles using the same conditions as in the first round. This gives a product of 222bp. All PCR experiments were performed twice using the same cDNA and either no RNA or RNA from HL60 cells were used as negative controls. RNA integrity was also assessed by the successful amplification of normal *ABL* transcripts (Hernandez *et al*, 1990) (see Chapter 3.2.4).

4.2.5 Sensitivity

In order to determine the lower limits of reproducible transcript detection, MNCs from a known case of AML M2 at diagnosis with t(8;21) and 94% myeloblasts were serially diluted in normal peripheral blood MNCs. RNA was extracted from mixtures containing one t(8;21) cell in 10, 10^2, 10^3, 10^4, 10^5 or 10^6 *AML1-ETO* negative MNCs and the RT-PCR assay performed as described above.

4.2.6 Sequencing

Specificity of PCR products was confirmed by direct sequencing. Nested PCR products were electrophoresed through low melting point agarose to remove contaminating oligonucleotides, the required band excised, cleaned and sequenced using a ^{32}P end-labelled primer A2 with the fmol™ DNA Sequencing System (Promega) (see Chapter 2).

4.3 Results

4.3.1 Sensitivity

It was found that this nested RT-PCR technique could reliably detect one leukaemic cell expressing *AML1-ETO* fusion transcripts present in 10^5 peripheral blood MNCs (Figure 4.2).

4.3.2 Incidence of t(8;21) by cytogenetics

Of the 396 AML patients studied by conventional cytogenetics, 32 (8.1%) were found to have a t(8;21) (Table 4.2). The majority of these were M2 patients (81.3%), with 3 cases each of subtypes M1 and M4.

4.3.3 Incidence of *AML1-ETO* fusion transcripts

All 396 patients had detectable normal *ABL* transcripts indicating integrity of the RNA used. *AML1-ETO* fusion transcripts were detected by RT-PCR in 51/396 (12.9%) of patients studied. All 32 cases of t(8;21) detected by conventional cytogenetics were PCR positive. A further 19 cases were PCR positive (M1, n=6; M2, n=11; M4, n=1; M5, n=1) although no structural abnormalities of either chromosome 8 or 21 had been reported on karyotype analysis. Review of the cytogenetic slides from 14 of these cases revealed poor quality metaphase spreads in 2 cases and good quality metaphase spreads in 12. Further review of these 12 cases confirmed the absence of gross abnormalities of either chromosome 8 or 21.

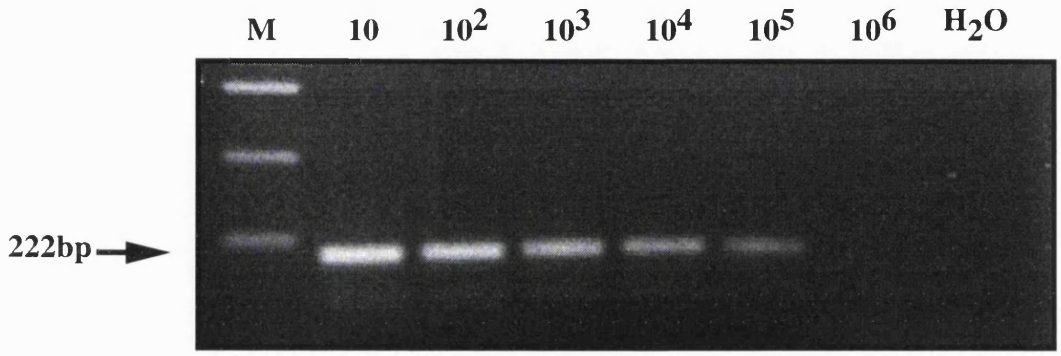


Figure 4.2 Sensitivity of *AML1-ETO* RT-PCR. Number of MNC per t(8;21) cell. M: size markers.

Table 4.2 Results of cytogenetic and molecular screening

FAB type	Number studied	t(8;21) detected by cytogenetics	*AML1-ETO* detected by RT-PCR	*AML1-ETO* but not t(8;21)	Frequency in AML*	Observed frequency of AML1-ETO**	Calculated frequency of *AML1-ETO*
M0	5	0 (0%)	0	0	<5%	0%	0%
M1	85	3 (3.5%)	9	6	20%	10.6%	2.1%
M2	131	26 (19.9%)	37	11	30%	28.2%	8.5%
M3	49	0 (0%)	0	0	10%	0%	0%
M4	79	3 (3.8%)	4	1	20%	5.1%	1.3%
M5	35	0 (0%)	1	1	10%	2.9%	0.3%
M6	8	0 (0%)	0	0	<5%	0%	0%
M7	4	0 (0%)	0	0	<5%	0%	0%
Total	396	32 (8.1%)	51 (12.9%)	19 (4.8%)			12.2%

* Frequencies based on data from Heim & Mitelman (1995)
** Data from this study

4.3.4 Specificity of RT-PCR products

Specificity of the PCR products was confirmed in 4 cases by sequencing the semi-nested product across the molecular fusion site. Two patients had cytogenetic and molecular evidence of t(8;21) and 2 had molecular evidence only. All 4 patients had the expected *AML1-ETO* sequence.

4.4 Discussion

The cytogenetic profile of AML patients at diagnosis has been found to be one of the most important prognostic factors in this disease. As AML patients with a t(8;21) at diagnosis have a relatively good prognosis in response to high dose chemotherapy (Bloomfield *et al*, 1998; Byrd *et al*, 1999; Grimwade *et al*, 1998) detection of the molecular fusion created by this translocation is important in order to assign patients to the correct treatment protocol. In the present study 12.9% (51/396) of all AML patients analysed had molecular evidence of the *AML1-ETO* fusion. Of these, over one third (19/51, 37%) had not been detected by conventional cytogenetic techniques. In some cases this may have been due to poor quality metaphase preparations, but in the majority of cases the translocation was not detectable at the gross karyotypic level. The failure to detect this abnormality was highest in those cases deemed to be of the morphological M2 subtype (n=11) but *AML1-ETO* transcripts were also detected in cases with M1 (n=6), M4 (n=1) and M5 (n=1). To calculate the proportion of AML cases that have an *AML1-ETO* fusion, the observed frequencies have been adjusted for the incidence of each subtype based on the data of Heim & Mitelman (1995) (Table 4.2). Overall, this indicates that 12.2.% of all AML cases would be expected to have an *AML1-ETO* fusion. The

adjusted data suggest that non-M2 subtypes account for 27.5% (14/51) of cases with *AML1-ETO* transcripts detected by RT-PCR.

Previous reports have suggested that masked rearrangements of *AML1-ETO* without cytogenetic evidence of the t(8;21) are restricted to cases of the M2 subtype (Maruyama *et al*, 1993; Andrieu *et al*, 1996). A correlation between the characteristic morphology of t(8;21) myeloblasts (distinctive cytoplasmic granulation and vacuolation, prominent Auer rods and occasional eosinophilia) and the presence of *AML1-ETO* transcripts has been described, awareness of which could increase the detection rate of the molecular fusion (Nucifora *et al*, 1994). RT-PCR screening of RNA from large numbers of AML patients by Krauter *et al* (1998) (2 t(8;21)-/*AML1ETO*+/140 cases studied, 1.4%), Mitterbauer *et al* (1998) (2/204, 1.0%), Pallisgaard *et al* (1998) (3/102, 2.9%) and Rowe *et al* (2000) (2/412, 0.5%) has since verified the existence of this phenomenon in a number of patients but at a lower incidence than reported here (19/396, 4.8%). This apparent discrepancy may be the result of a number of factors: (1) Deterioration of sample and thus RNA quality during transit in multicentre trials. (2) Differences in the technical parameters of the RT-PCR methodologies employed such as primer sequences, amplification conditions or number of cycles used. (3) Selection of patients. In the studies by Mitterbauer *et al* (1998), Pallisgaard *et al* (1998) and Rowe *et al* (2000) no ages of the patients studied are given. As the t(8;21) occurs more frequently in children and younger adults with AML than in older patients (Leblanc & Berger, 1997), then the incidence of cryptic rearrangements of *AML1-ETO* may also be raised in this group.

Creation of functional fusion genes in the absence of cytogenetic evidence for the associated translocation has been previously described in a number of cases of AML with either cryptic *PML-RARα* (Lafage-Pochitaloff *et al*, 1995; Grimwade *et al*, 1997), *CBFB-*

MYH11 (Pirc-Danoewinata et al, 2000) or *MLL-AF4* (Uckun *et al*, 1998) rearrangements. FISH analysis using *AML1* and *ETO* probes in two cases of AML with complex variant t(8;21) has shown that insertion of either a segment of *AML1* into chromosome 8 or a part of *ETO* into chromosome 21 can result in a transcriptionally active fusion gene (Taviaux *et al*, 1999) possibly via a combination of translocation and insertion events. These complex rearrangements involving *AML1* are not restricted to AML. Reddy *et al* (2000) have recently described a child with ALL in whom insertion of *AML1* into chromosome 12 created a *TEL-AML1* fusion gene. It was identical to that created by the t(12;21)(p13;q22) which joins nearly all of *AML1*, retaining the DNA binding and transactivation domains, to the 3' end of the *TEL* gene.

In a recent study to detect either *AML1-ETO* transcripts by RT-PCR or the resulting protein by Western blotting, Sarriera *et al* (2001) found evidence of a masked t(8;21) in 8/104 (7.7%) patients. Comparison of CR rates between 5 of these patients and 21 with karyotypic evidence of t(8;21) suggested a substantially reduced response to high dose chemotherapy in the former group. However, because of the small number of patients considered in this study, cautious interpretation of these results is necessary. Preliminary survival analysis has been performed by Dr K. Wheatley (Clinical Trials Service Unit, Radcliffe Infirmary, Oxford) on the 19 patients in the present study with only molecular evidence of *AML1-ETO* transcripts . Eighteen (95%) achieved a CR which is similar to the 98% CR rate seen in patients with cytogenetic t(8;21) recently entered into the UK MRC AML 10 trial (Grimwade *et al*, 1998). Although the CR rate is high, because of the relatively small number of patients studied, it is not significantly different from the 88% CR rate seen in patients with normal cytogenetics entered into the MRC AML10 trial (Hann *et al*, 1997). Similarly, the disease-free survival and overall survival of the 19 patients with molecular evidence only of *AML1-ETO* were not significantly different

from either those patients with normal cytogenetics or those with t(8;21), although as yet this analysis is limited by the relatively short follow-up of patients (range 5 - 24 months). Possible further investigations would include screening a larger number of patients with longer follow-ups.

The detection of *AML1-ETO* transcripts in AML patients at diagnosis affords the opportunity to use this as a marker of MRD. As the fusion gene is detectable by qualitative RT-PCR in remission patients up to 6 years post alloBMT (Jurlander *et al*,1996), it has become necessary to develop quantitative assays to monitor disease progression. Competitive (Muto *et al*, 1996; Tobal *et al*, 2000) and 'real time' (Marcucci *et al*, 1998) strategies have been shown to be of value in determining response to therapy and in the early detection of relapse. Both approaches are comparable in their sensitivity, linearity and reproducibility, irrespective of whether serial dilutions of plasmids, cell lines or samples from t(8;21) positive AML patients at different stages of the disease were analysed (Wattjes *et al*, 2000). However, the 'real-time' methodology offers some technical advantages because of its automated nature. Quantitative assessment of MRD is also possible by FISH techniques. Paskulin *et al* (1998) reported a method using probes that identify chromosomes 8 and 21 in which a fusion signal was detected in addition to the normal 8 and 21 alleles in 20/20 t(8;21) AML patients at diagnosis, with no false positives among 5000 normal cells. Probe combinations from the *AML1* gene flanking or overlapping the breakpoint region have been investigated which have similar detection rates (Tanaka *et al*, 1999). Using larger genomic breakpoint probes, Hagemeijer *et al* (1998) were able to detect the onset of relapse in both the bone marrow and peripheral blood of 14 patients with t(8;21) AML by counting up to one thousand interphases. As cytogenetic analysis belongs to the first line of investigation in acute leukaemia, FISH techniques are of value where no further cellular material is available for RNA extraction.

The results of this study suggest that if the presence of the t(8;21) in AML is to be used to direct treatment decisions such as chemotherapy versus BMT, then it should be considered necessary to screen all new cases of AML for the presence of *AML1-ETO* fusion transcripts. This would increase detection of patients with apparently good-risk disease. Correct identification of the fusion gene would identify those patients who might be eligible for therapies that target either the abnormal mRNA transcript or protein, such as HDAC inhibitors (Kosugi *et al*, 1999), which have been shown *in vitro* to relieve ETO-mediated repression and induce differentiation of AML1-ETO leukaemia cells (Wang *et al*, 1999). Another potential therapeutic approach is the use of ribozymes which are oligoribonucleotides with sequence-specific cleavage activity of target RNA which therefore suppress gene expression. Ribozymes have been shown to effectively cleave *AML1-ETO* RNA and induce apoptosis of t(8;21) bearing cells *in vitro* (Matsushita *et al*, 1999; Szyrach *et al*, 2001).

Chapter 5

TMA/HPA analysis of *BCR-ABL* in CML

5.1.1 Introduction

The introduction of PCR methodologies has allowed the detection of numerous fusion genes created by chromosomal translocations in both lymphoid and myeloid haematological malignancies. Detection of these rearrangements is not only important in the correct assignment of disease entities/subtypes allowing appropriate and optimal treatments, but also provides a marker with which it is possible to monitor the patient throughout the clinical course of their disease. This monitoring may provide useful information of a patients' response to therapy and may also be of value in heralding an impending relapse, thus allowing clinical intervention before overt haematological or clinical relapse.

In this chapter, results are presented on the evaluation of a novel amplification and detection strategy for determining the *BCR-ABL* levels associated with chronic myeloid leukaemia (CML).

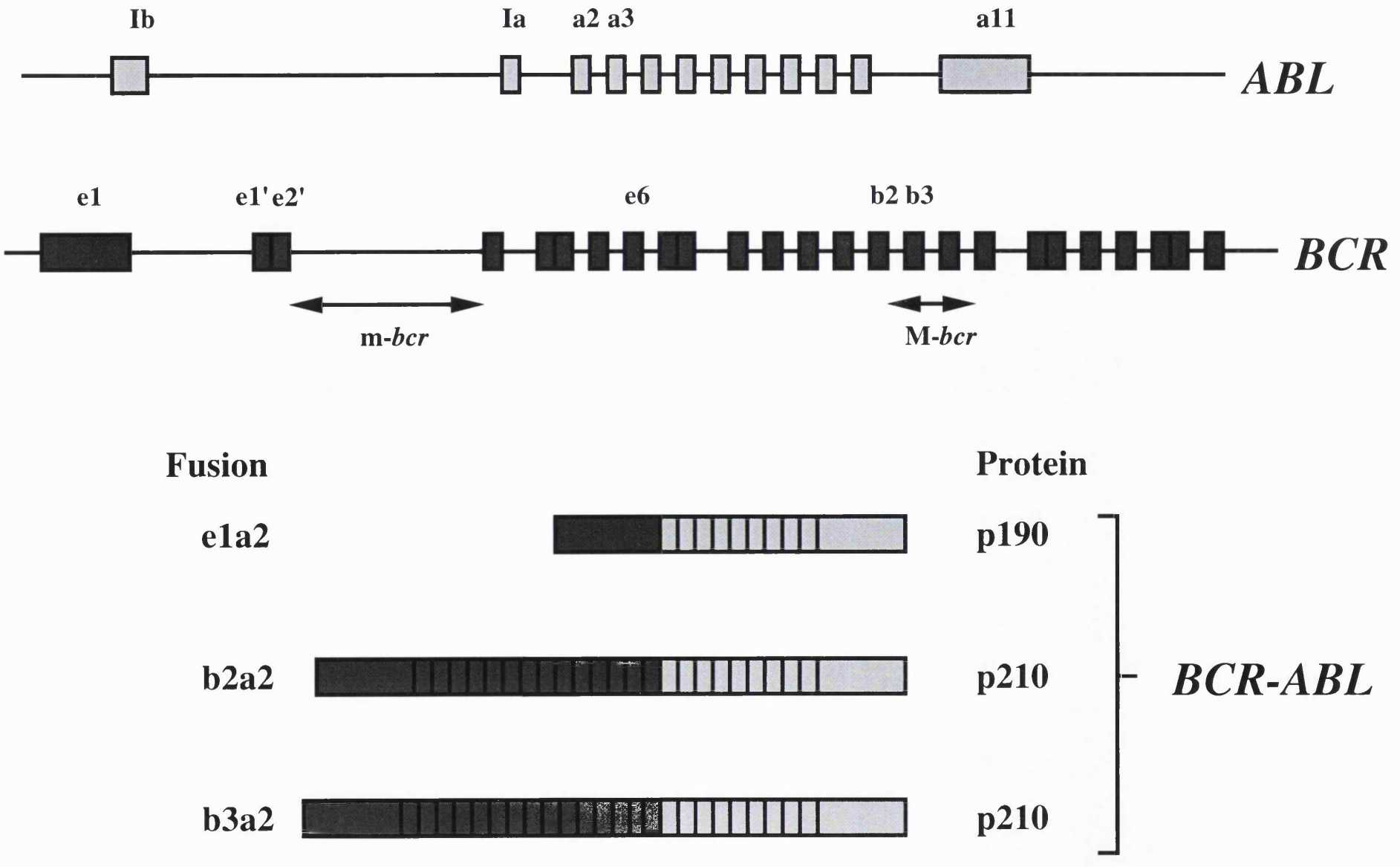
5.1.2 CML

CML is a clonal myeloproliferative expansion of transformed primitive haemopoietic progenitor cells in the bone marrow. It involves the myeloid, monocytic, erythroid, megakaryocytic and B-lymphoid cell lineages. CML was the first human disease in which a specific abnormality of the karyotype, the Philadelphia (Ph) chromosome, could be linked to the pathogenetic events of leukaemogenesis (Nowell & Hungerford, 1960). The Ph chromosome is the derived chromosome 22 resulting from the balanced translocation t(9;22)(q34;q11) between the long arms of chromosomes 9 and 22. It was also among the first malignant disorders in which therapy with a biological agent (interferon) was found to suppress the leukaemic clone and prolong survival (Kurzrock *et al*, 1998). CML has an incidence of 1 to 2 cases per 100,000 people per year and accounts for approximately 15% of adult leukaemia. The median age of patients at presentation is 45-55 years. Most cases (85%) are diagnosed in the chronic phase. Common findings at presentation are fatigue, weight loss,

bleeding, purpura, splenomegaly, leucocytosis, anaemia and thrombocytosis. In most cases, three to five years after onset CML progresses to the accelerated and blast crisis phases, often characterised by an increase in bone marrow blast cell numbers which are phenotypically similar to those of either AML or ALL and cytogenetic clonal evolution.

With the refinement of cytogenetic banding techniques, a balanced t(9;22)(q34;q11) translocation can now be demonstrated by cytogenetics in up to 95% of CML patients at presentation (Sawyers *et al*, 1999). This translocation is also observed in about 25% cases of ALL and rarely in AML. The Ph chromosome fuses a 3' segment of the *ABL* gene from chromosome 9q34 to the 5' part of the *BCR* gene on chromosome 22q11, creating a hybrid *BCR-ABL* gene that is transcribed into chimeric *BCR-ABL* mRNA. Breakpoints usually occur 5' of exon 2 such that *ABL* exons 2 to 11 are transposed into the major breakpoint cluster region (M-bcr) between exons 12 and 15 of the *BCR* gene on chromosome 22. The breakpoint locations within *BCR* fall either 5' between exons b2 and b3, or 3' between exons b3 and b4 (Figure 5.1). The 8.5 kb mRNA is translated into a chimeric protein of 210 kDa. In ALL the breakpoint on chromosome 22 falls within a segment called the minor breakpoint cluster region (m-bcr). Splicing out exons e1' and e2' forms a *BCR-ABL* transcript that is translated into a smaller fusion protein of 190 kDa (Faderl *et al*, 1999a) (Figure 5.1). Evidence for the role of *BCR-ABL* transcripts as central mediators of myeloid proliferation and transformation in CML comes from experiments where these transcripts cause factor-independent and leukaemogenic cell growth in haemopoietic cell lines and can also generate a syndrome in mice that closely resembles human CML (Daley *et al*, 1990; Heisterkamp *et al*, 1990). ABL proteins are tyrosine kinases that have important roles in signal transduction and cell growth (Wang, 1993) and the various structural alterations of ABL and BCR facilitate the leukaemic transformation by *BCR-ABL*. For example, the structure of the p210 BCR-ABL protein allows multiple protein-protein interactions and suggests the involvement of diverse intracellular signalling pathways.

Figure 5.1 Exon structure of *ABL*, *BCR* and *BCR-ABL* genes



During the chronic phase of CML, cytoreductive therapy with hydroxyurea and/or apheresis is required in most patients to avoid thrombotic complications that can result from a high circulating level of neutrophils. After stabilisation, eligible patients may elect to undergo high dose chemotherapy that destroys the normal bone marrow haemopoietic cells followed by allogeneic bone marrow (alloBMT) or stem cell transplantation (SCT). The success of allogeneic transplantation is age-dependent, being significantly lower in patients over the age of 40 years, primarily because of higher treatment related mortality. Other variables necessary to consider for this procedure include the disease stage and the level of donor-recipient HLA matching which limits the use of this option to approximately 20% of CML patients. A correlation was observed between the incidence of graft versus host disease (GVHD) and long term disease free survival (Weiden *et al*, 1981). This graft versus leukaemia (GVL) effect became evident when transplants were performed with T cell depleted bone marrow, successfully reducing mortality from GVHD but increasing the relapse risk (Goldman *et al*, 1988). These results implicated T lymphocytes as critical in the success of alloBMT. Subsequently threshold levels of donor lymphocyte infusions (DLI) have been shown to induce remissions in the absence of chemotherapy (Mackinnon *et al*, 1995). Interferonα (IFNα) therapy is currently the first line treatment of choice in those patients ineligible for alloBMT (Allan *et al*, 1995) and can induce haematological and cytogenetic remissions in CML patients in chronic phase.

As the outcome for CML patients has improved with the introduction of the aforementioned treatments together with the recent development of agents that specifically inhibit the *BCR-ABL* tyrosine kinase activities (Druker *et al*, 2001a & b), so the t(9;22) translocation has been used increasingly as a marker of minimal residual disease (MRD). Cytogenetic analysis is still necessary in the monitoring of CML patients to detect other structural and numerical abnormalities apart from the t(9;22) which may herald clonal evolution or transformation of the leukaemia. As only 20-50 metaphases are analysed routinely, karyotypic analysis is a relatively insensitive means of monitoring MRD during IFNα therapy or after alloBMT (Yee *et al*, 1999). FISH and Southern blotting too lack the sensitivity required, with a detection

limit of approximately one t(9;22)+ cell in 100 t(9;22)- cells. Therefore most studies have relied on detection of *BCR-ABL* transcripts. However, the sensitivity of qualitative RT-PCR techniques varies, and as many patients remain *BCR-ABL* PCR positive for some considerable time following alloBMT, the usefulness of these types of studies in predicting clinical outcome remains controversial (Faderl *et al*, 1999b). In order to address this issue, quantitative methods have been introduced. For example, in competitive PCR a synthetic competitor molecule is constructed which contains the sequences necessary for *BCR-ABL* PCR but which produces a fragment of slightly larger size than the *BCR-ABL* under investigation, i.e. b2a2 or b3a2 fusion products. Increasing amounts of competitor are added to a series of PCR reactions from patient cDNA and comparison of the resulting product intensities is used to estimate the level of transcript in the patient sample. This method has been shown to detect a fall in *BCR-ABL* levels after alloBMT (Thompson *et al*, 1992; Cross *et al*, 1993; Lion *et al*, 1993) and an elevation prior to relapse (van Rhee *et al*, 1994; Lin *et al*, 1996) and has been used to determine the rate of response to DLI once relapse has occurred (Raanani *et al*, 1997). However, the method requires multiple PCR reactions for each sample, which is time consuming, and reproducibility is not good. More recently, an alternative approach using 'real-time' PCR has been introduced whereby the number of *BCR-ABL* molecules is estimated on the basis of the number of amplification cycles required to reach a threshold level of detection. Studies using 'real-time' quantification have been used to demonstrate falls in *BCR-ABL* levels post alloBMT and after treatment with DLI for relapse (Mensink *et al*, 1998; Preudhomme *et al*, 1999; Eder *et al*, 1999). The method can detect one *BCR-ABL* positive K562 cell in the presence of 10^4 - 10^5 *BCR-ABL* negative cells, but it requires sophisticated and expensive equipment.

An evaluation was therefore performed of an RNA-based method of quantifying *BCR-ABL* transcripts in samples from peripheral blood or bone marrow of CML patients in which multiple RNA copies of the specified transcript are produced by Transcription-Mediated Amplification (TMA). They are then hybridised to acridinium ester (AE)-labelled DNA

probes and the resulting chemiluminescent signal quantified in the sensitive Hybridisation Protection Assay (HPA) (Arnold *et al*, 1989; Nelson, 1998).

5.2 Principles of TMA and HPA

5.2.1 Transcription mediated amplification (TMA)

The dynamic TMA process can be simplified in the following steps. Step 1: The *ABL* sequence-specific primer, which also contains the promoter sequence for RNA polymerase at its 5' end, binds to the 3' end of the target RNA; Step 2: Reverse transcriptase (RT) creates DNA copy of RNA target; Step 3: The RNA in the resulting RNA:DNA heteroduplex is degraded by the RNAse H activities of the RT. Step 4: A 5' *ABL* or *BCR* primer binds to the DNA; Step 5: RT completes the double-stranded DNA template in which both strands now include the RNA polymerase promoter sequence; Step 6: RNA polymerase initiates transcription producing 100-1,000 copies of either *BCR-ABL* or *ABL* amplicon; Step 7: Either the 5' *ABL* or *BCR* primer binds to the RNA amplicon; Step 8: RT creates an RNA:DNA heteroduplex; Step 9: RNAse H degrades the RNA strand; Step 10: The *ABL* promoter-primer binds to the newly synthesised DNA; Step 11: RT creates double-stranded DNA which is then available as template for Step 6 (Figure 5.2). As each of the created double-stranded DNA template molecules can lead to the exponential production of 100-1,000 RNA amplicons, the expansion can result in billions of amplicons produced within one hour.

5.2.2 Hybridisation protection assay (HPA)

In the HPA, sequence-specific oligonucleotide probes labelled with an acridinium ester (AE) are allowed to hybridise to the amplicons produced in the TMA reaction (Figure 5.3). Unhybridised probe is removed by rapid hydrolysis and the chemiluminescent signal from the remaining, more slowly hydrolysed, probe-amplicon hybrids is measured in a

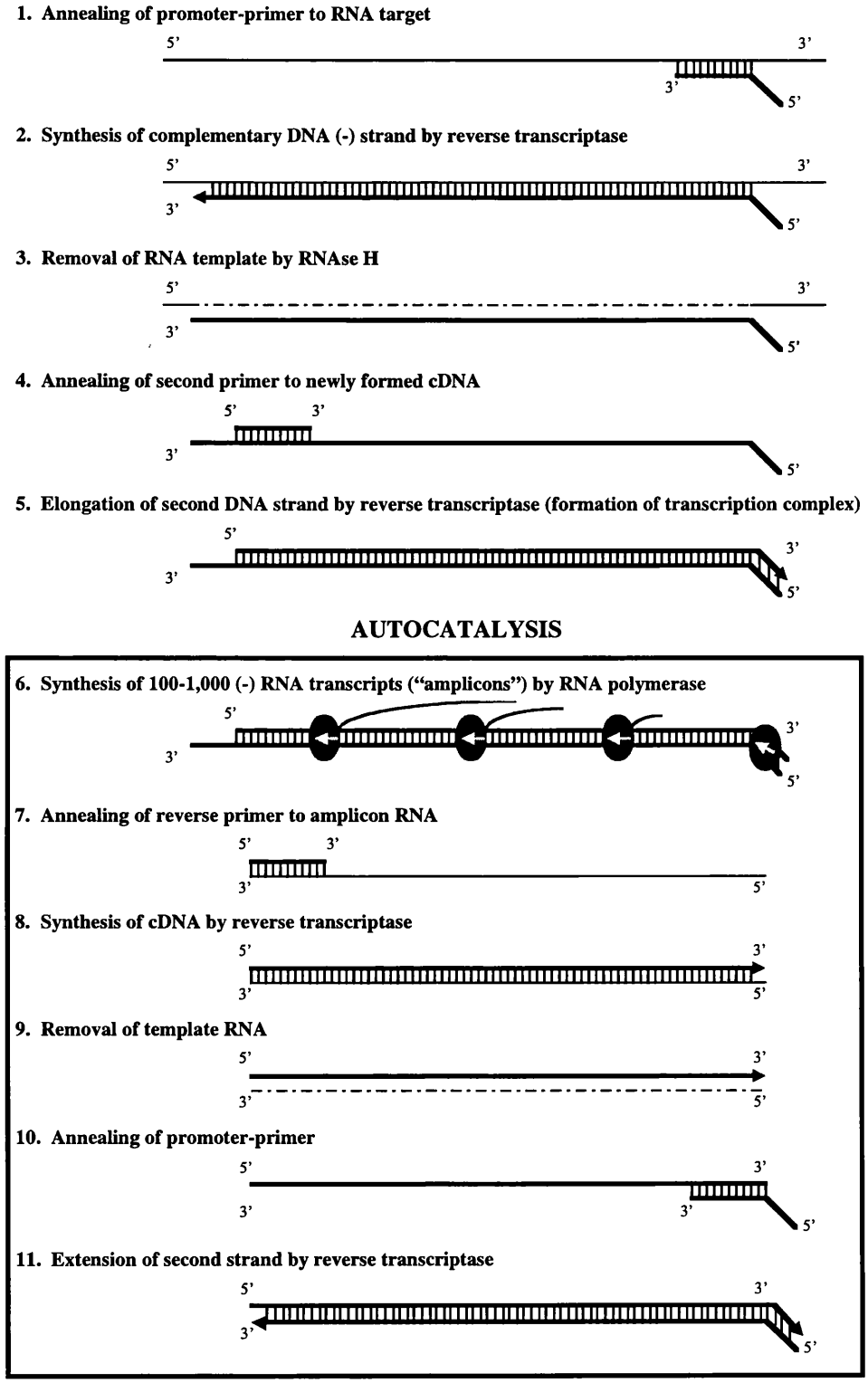


Figure 5.2 Transcription-mediated amplification.

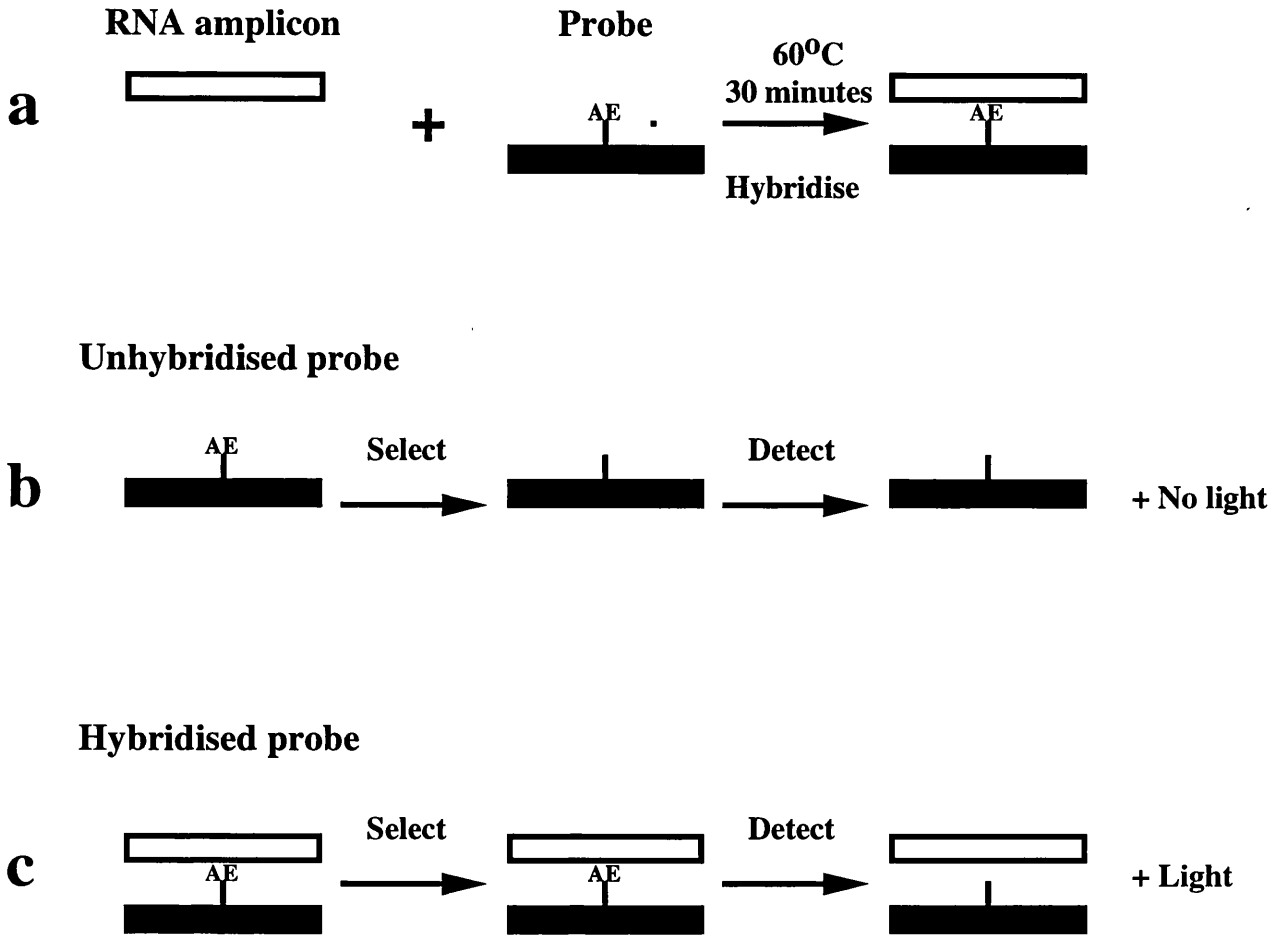


Figure 5.3 Detection of amplicon with DNA probes and the Hybridisation Protection Assay (HPA). (**a**) An excess of acridinium ester (AE)-labelled probe is added and allowed to hybridise to *BCR-ABL* or *ABL* target sequences within the amplicons produced by the TMA reaction. Separation of hybridised from unhybridised probe is achieved by addition of a selection reagent which hydrolyses the AE on the unhybridised probe. (**b**) No light is emitted from the hydrolysed unhybridised probe. (**c**) The AE on the hybridised probe is protected within the double helix and is not hydrolysed by the selection reagent. Light is therefore emitted and detected by the luminometer.

luminometer. The number of RNA amplicons produced is directly proportional to the number of target molecules in the starting sample, and only one molecule of AE-labelled probe can bind to an RNA amplicon. The chemiluminescent signal obtained is therefore a measure of starting template concentration.

5.3 Materials and methods

5.3.1 Patients

RNA was analysed from 20 haematologically normal controls (BM n=10, PB n=10) and from 20 patients with CML in chronic phase (BM n=10, PB n=10). A further 6 CML patients were analysed who had relapsed following alloBMT and had been treated with DLI.

5.3.2 Cytogenetics

Cytogenetics were performed as described in Chapter 3.2.2.

5.3.3 Sample preparation

RNA sample preparation was performed as described in Chapter 3.2.3.

5.3.4 RT-PCR analysis

Reverse transcription for *BCR-ABL* transcripts was performed as described in Chapter 3.2.4 using primer D (Table 5.1). For the first round of PCR, 2 µl of the reverse transcription reaction was used in a reaction total volume of 20 µl with final concentrations of 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.5 units of Taq DNA polymerase and 200 ng of each primers B and I (Table 5.1). After 5 minutes at 95°C, 30 cycles were performed consisting of 95°C (30 seconds), 55°C (30 seconds) and 72°C (30 seconds) followed by a

Table 5.1 RT-PCR primer, TMA primer and AE-labelled HPA probe sequences for the amplification and detection of *BCR-ABL* and *ABL* transcripts.

Primer/probe	Sequence (5'→3')
BCR-ABL RT-PCR primer B	GAAGAAGTGTTTCAGAAGCTTCTCCC
BCR-ABL RT-PCR primer I	GCTTCACACCATTCCCCATT
BCR-ABL RT-PCR primer E	TGTGATTATAGCCTAAGACCCGGAG
BCR-ABL RT-PCR primer J	GTGAAACTCCAGACTGTCCACAGCATTCCG
ABL TMA promoter primer	TAAATTAATACGACTCACTATAGGGAGACTCAGACCCTGAGGCTCAAAGTCAGA
BCR TMA reverse primer	GACCAACTCGTGTGTGAAACTCCA
ABL TMA reverse primer	CAAAGGAAGCAGGGAAGAAGG
BCR-ABL HPA AE-probe	GACTGTCCACAGCATTCCGCTGACC
ABL HPA AE-probe	GTGGAACATGAAGCCCTTCAGCGG

final extension at 72°C (5 minutes). This amplifies first round *BCR-ABL* PCR products of 339 bp for b2a2 transcripts and/or 413 bp for b3a2 transcripts. Two µl of the first round product was diluted in 498 µl of H_2O and a second round of PCR performed with 2 µl of this as template in a 20 µl final volume PCR. Reagent concentrations were as in round one but using the nested primers E and J (Table 5.1). Cycling conditions were the same as in round one but with an annealing temperature of 60°C and the number of cycles reduced to 25. This amplified a second round *BCR-ABL* PCR product of 254 bp for b2a2 transcripts and/or 329 bp for b3a2 transcripts. RT-PCR for *ABL* transcripts was performed as described in Chapter 3.2.4.

5.3.5 TMA

Test RNA (100 ng unless otherwise stated) in a total volume of 25 µl was mixed with 50 µl core amplification reagent (Gen-Probe Inc., San Diego, CA. U.S. patent 5,888,779) containing 2X amplification buffer (2 mM each dNTP, 8 mM each rNTP, 80 mM Tris-HCl pH 7.5 @ 25° C, 50 mM $MgCl_2$, 35 mM KCl, 10% (w/v) polyvinylpyrrolidone) and 15 pmols each promoter-primer and reverse primer for either *BCR-ABL* or *ABL* (Table 5.1) in a 12 x 75 mm polypropylene tube and incubated at 60° C for 10 minutes under oil to allow denaturation of the RNA. The mixture was then cooled to 42° C for 5 minutes before adding 25 µl of enzyme mix containing MMLV reverse transcriptase (2,000 units/assay) and T7 RNA polymerase (2,000 units/assay) in 8 mM HEPES, pH 7.5, 50 mM N-acetyl-L-cysteine, 0.04 mM zinc acetate, 80 mM trehalose, 140 mM Tris-HCl pH 8.0 @ 25° C, 70 mM KCl, 1 mM EDTA, 0.01% (w/v) phenol red, 10% (v/v) Triton® X-100 and 20% (v/v) glycerol) and incubation continued for a further 60 minutes at 42° C.

5.3.6 HPA

One hundred microlitres of probe mix containing 7.5 nM AE-labelled oligonucleotide probe complementary either to the *BCR* exon b2 for *BCR-ABL* products or to an *ABL* 1b sequence

(Table 5.1) for *ABL* products in hybridisation buffer (100 mM lithium succinate pH 4.7, 2% (w/v) lithium lauryl sulphate, 15 mM aldrathiol-2, 1.2 M lithium chloride, 20 mM EDTA, 20 mM ethylene glycol-bis-(-amino ethyl ether) N, N, N', N'-tetracetic acid (EGTA), 3% ethanol, Gen-Probe Inc.), was then added to the TMA reaction, the mixture vortexed and incubated for 30 minutes at 60° C. To remove unhybridised probe, 300 µl hydrolysis buffer (600 mM sodium tetraborate pH 8.5, 1% (v/v) Triton® X-100) was then added and the sample incubated at 60° C for a further 10 minutes. After cooling the tubes were placed in a luminometer with an automated reagent-injection system and 200 µl Reagent 1 (1ml/l H_2O_2, 1 mM nitric acid) and 200 µl Reagent 2 (1M NaOH) were added. The resulting chemiluminescence was integrated for 2 seconds and recorded as relative light units (RLU). Each sample was tested in triplicate and the mean result after subtraction of the background (i.e. no RNA) level calculated. TMA primers and HPA probes were manufactured and supplied by Gen-Probe Incorporated, San Diego, CA, USA.

5.3.7 Statistical analysis

Simple regression analysis was used to verify the linearity of calibration curves. Student's t-test was employed to analyse the difference in both *BCR-ABL* and *ABL* levels of CML and control patient samples: a p value of less than 0.05 was deemed to be significant. Coefficients of variation (CV) were calculated to assess technical variation in multiple sample analysis.

5.4 Results

5.4.1 Technical evaluation of TMA/HPA

5.4.1.1 Sensitivity and linearity

In order to evaluate the sensitivity of the technique and linearity of the increase in RLUs obtained with increasing template concentration, the *BCR-ABL* and *ABL* signals from varying

amounts (0 – 500 ng) of total RNA from the *BCR-ABL* positive cell line K562 were measured as described above. In addition, RNA was extracted from mixtures containing 1 K562 cell in 10, 10^2, 10^3, 10^4, 10^5 or 10^6 HL60 cells (*BCR-ABL* negative) and 100 ng used to assay the *BCR-ABL* and *ABL* signals. In the *BCR-ABL* assay, levels above background were detected using greater than 0.005 ng K562 RNA (Figure 5.4a) and this correlated to the detection of 1 K562 cell in 10^4- 10^5 HL60 cells (Figure 5.4c). Regression analysis of the values obtained for 0.05, 0.5, 5 and 50 ng starting RNA gave r^2 values of 0.906-0.996 (mean 0.960, median 0.968) in 12 analyses. The *ABL* assay required greater than 0.5 ng K562 RNA to detect levels above background (Figure 5.4b), probably reflecting that there are fewer *ABL* than *BCR-ABL* transcripts per K562 cell (Wu *et al*, 1995). Regression analysis of the *ABL* values obtained for 0.5, 5, 50 and 500 ng starting RNA gave r^2 values of 0.887 - 0.992 (mean 0.960, median 0.961) in 12 analyses.

5.4.1.2 Reaction kinetics and product stability

In order to evaluate kinetics of the reaction and stability of the products created during TMA, samples from 3 patients with high, medium and low *BCR-ABL* values respectively were incubated at 42°C for times varying between 30 and 150 minutes, then HPA performed as described. Maximum levels were achieved after approximately 60 minutes of incubation in all 3 samples and remained stable for a period of at least a further 90 minutes (Figure 5.5a).

5.4.1.3 Reproducibility

Sample reproducibility was assessed using RNA from 3 patients with high, medium and low *BCR-ABL* levels and 3 with high, medium and low *ABL* levels respectively. Eight separate aliquots were prepared from each sample, each of 300ng, and assayed in triplicate (100ng/test) using the same batch of reagents. The results obtained are shown in Figure 5.5b. For the *BCR-ABL* assays, the CVs of the mean values from the 8 aliquots were 11%, 26% and 27% for the 3 samples respectively, and for the *ABL* assays they were 14%, 16% and

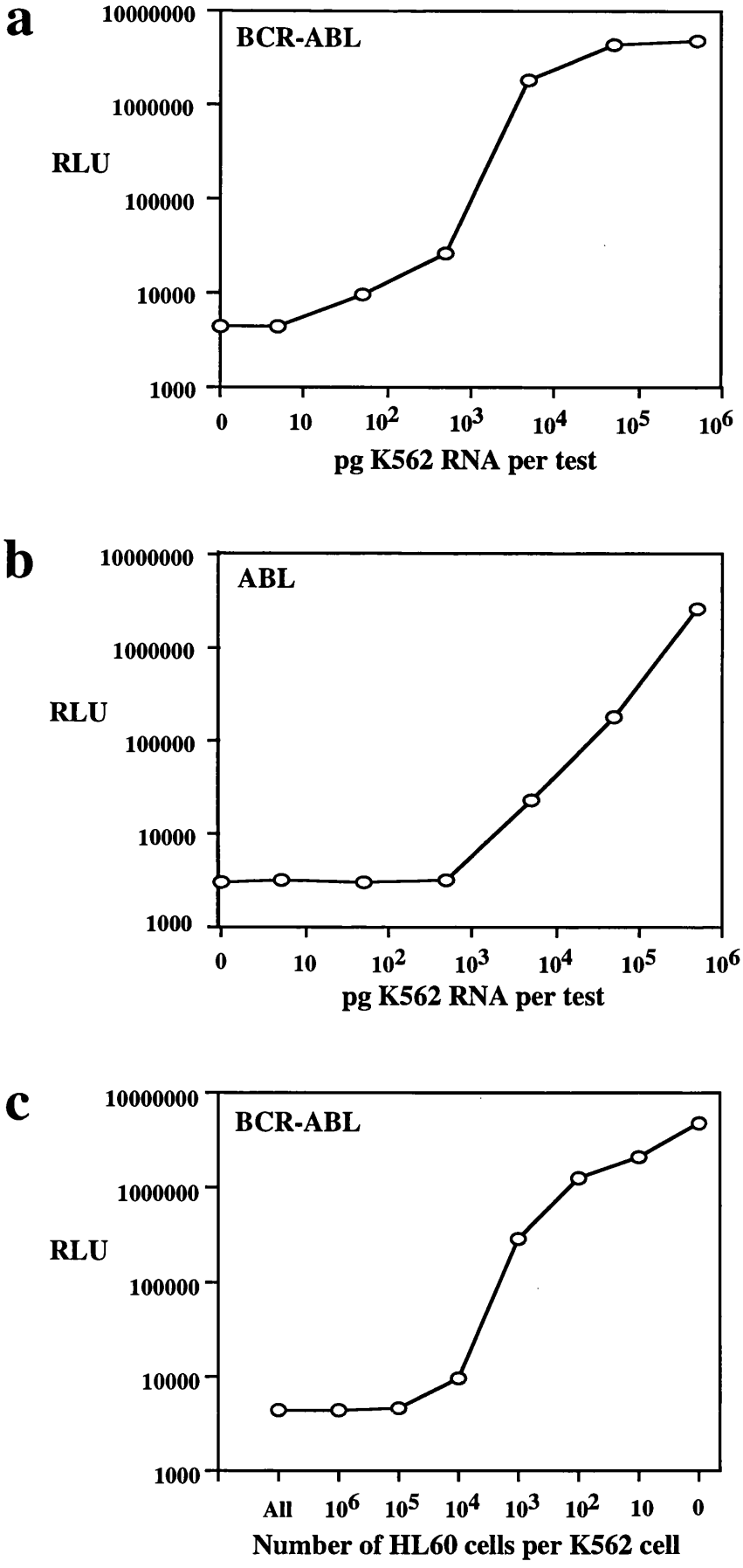


Figure 5.4 Linearity and sensitivity of assays. Increasing concentrations of K562 RNA were assayed for levels of (a) *BCR-ABL* and (b) *ABL* transcripts. (c) *BCR-ABL* levels were assayed in RNA from 1 K562 cell in the presence of increasing numbers of HL60 cells.

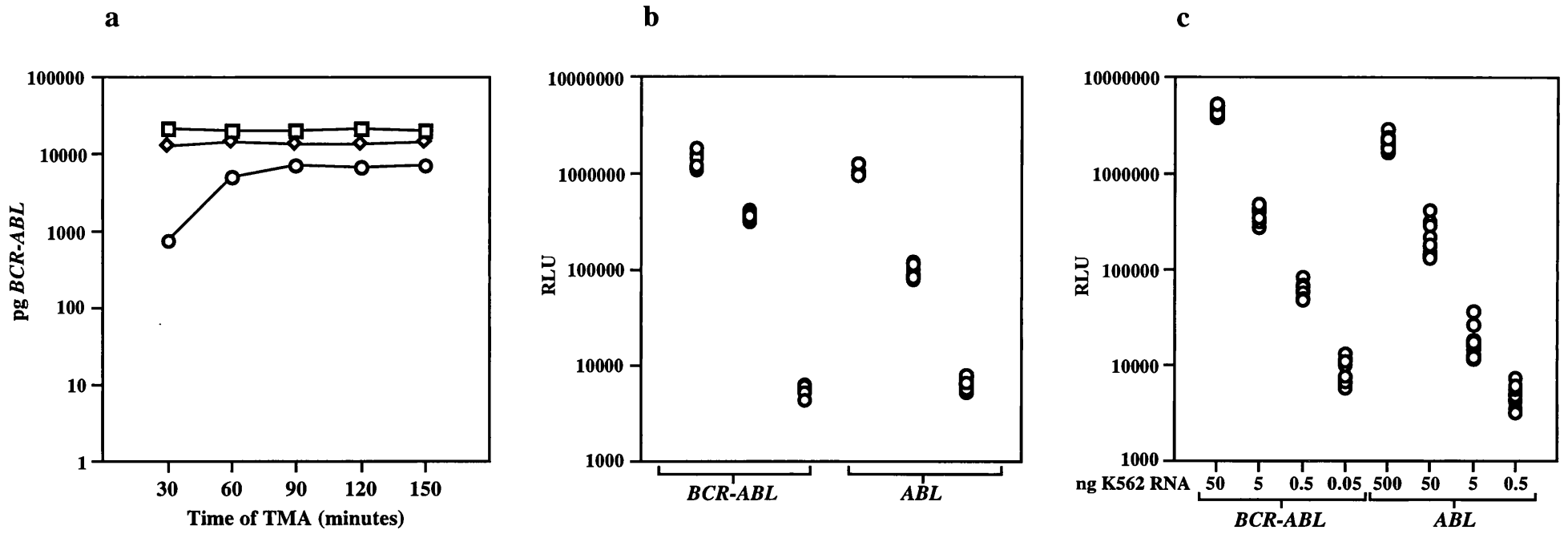


Figure 5.5 Assay kinetics and reproducibility. (**a**) Time course of TMA incubation using 3 patient samples with differing *BCR-ABL* levels. (**b**) Repeated analysis of samples with varying *BCR-ABL* or *ABL* levels. Each sample was assayed 8 times in triplicate. (**c**) Day to day variation of 4 different concentrations of K562 RNA assayed on 12 separate occasions.

26% respectively. Day to day technical variation was assessed using the data from 12 calibration curves created using 4 different dilutions of K562 RNA (Figure 5.5c). The CVs were 41%, 18%, 21% and 28% for 50, 5, 0.5 and 0.05ng RNA respectively in the *BCR-ABL* assay, and 35%, 17%, 19% and 24% for 500, 50, 5 and 0.5ng RNA in the *ABL* assay.

5.4.2 Analysis of CML patient samples

For each series of patient or control samples analysed, calibration curves using samples of 0.05, 0.5, 5 and 50 ng starting K562 RNA for *BCR-ABL* analysis and 0.5, 5, 50 and 500 ng for *ABL* analysis were constructed as described above and the slopes used to convert RLUs obtained into post-amplification quantities of RNA. One hundred nanograms template RNA was selected as the optimal amount to use per test as this gave post-amplification *BCR-ABL* and *ABL* levels within the linear range of the calibration curve for samples with varying levels of target.

5.4.2.1 Diagnosis

Levels of *BCR-ABL* and *ABL* transcripts were quantified in 100 ng total RNA from bone marrow (BM, n=10) or peripheral blood (PB, n=10) of 20 CML patients at presentation with clinical and cytogenetic t(9;22) disease in chronic phase, and from BM (n=10) or PB (n=10) of 20 haematologically normal controls (HNC) who all had a normal karyotype and were *BCR-ABL* negative by conventional RT-PCR analysis (Hernandez *et al*, 1990). In order to accommodate variations in RNA quality, *BCR-ABL* transcript levels were normalised by expressing the results as *BCR-ABL/ABL* ratios. The *BCR-ABL* level was not above background in 19 of the control samples and was at the background level at 0.005 ng in one BM sample (Figure 5.6a) whereas the *ABL* levels varied between 1.9 and 49.2 ng (median 14.1 ng) (Figure 5.6b). All 20 controls had *BCR-ABL/ABL* ratios $\leq$ 0.0001 (Figure 5.6c). In 18 of the CML patients (8 BM, 10 PB), *BCR-ABL* levels ranged between 0.2 and 43.0 ng (median 8.5 ng), *ABL* levels between 0.6 and 82.6 ng (median 14.9 ng) and the *BCR-*

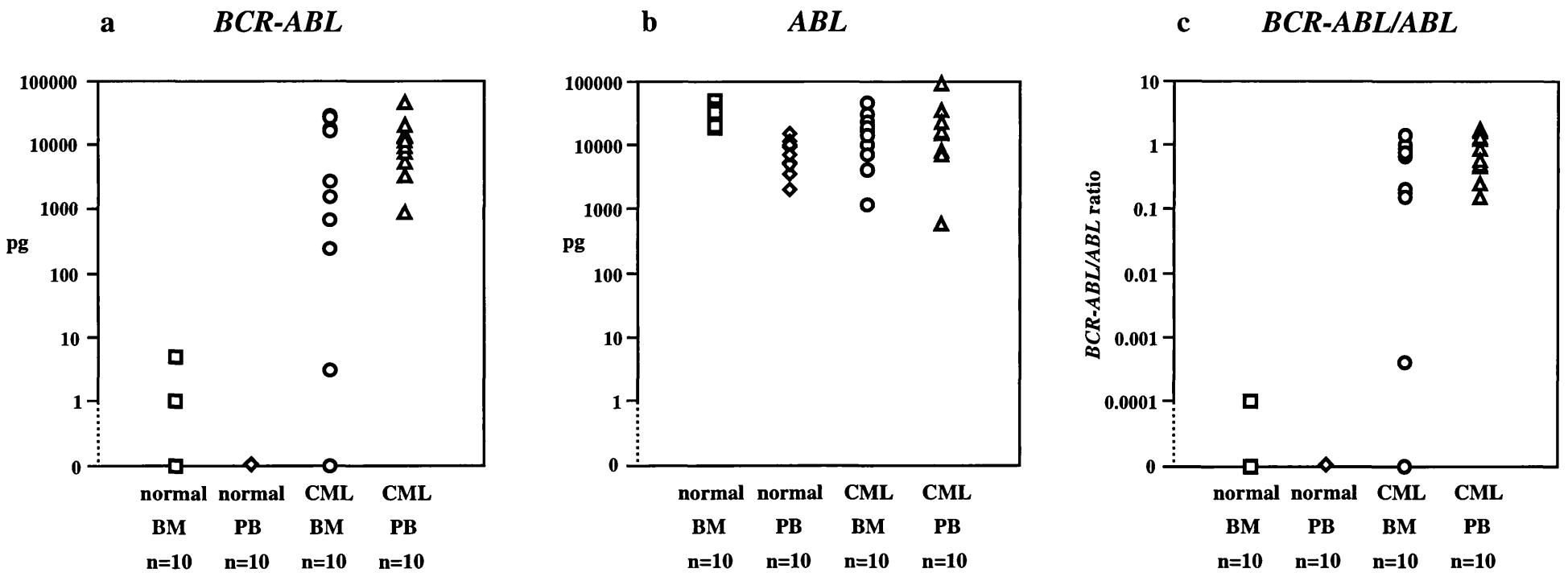


Figure 5.6 Post-amplification levels of (**a**) *BCR-ABL* and (**b**) *ABL* using RNA from 20 CML patients at diagnosis and 20 normal controls. (**c**) The corresponding *BCR-ABL/ABL* ratios.

ABL/ABL ratios between 0.15 and 1.59 (median 0.65). There was no significant difference between the *ABL* levels of the CML patients and normal controls, but *BCR-ABL* levels and *BCR-ABL/ABL* ratios were both significantly higher in the 18 CML patients than the controls (p=0.0001). In the two remaining CML samples, both from BM, the *BCR-ABL* levels were <0.005 ng (Figure 5.6a), the *ABL* levels were normal (7.1 and 14.0 ng respectively) and the *BCR-ABL/ABL* ratios were 0.0001 and 0.0004 (Figure 5.6c). These 2 samples were also *BCR-ABL* negative by conventional RT-PCR.

5.4.2.2 Post alloBMT

Sequential levels of *BCR-ABL* and *ABL* transcripts were quantified using 100 ng total RNA from PB (Figure 5.7a-c) or BM (Figure 5.7d-f) of six CML patients following alloBMT who all received DLI for haematological or molecular relapse. The 'absolute' level of *BCR-ABL* transcripts varied from patient to patient according to status and treatment, with a range of 0.013 – 53.3 ng, but the range of *ABL* levels was 0.7 – 123.8 ng (median 24.2 ng) which was similar to the control and diagnostic samples. *BCR-ABL/ABL* ratios ranged from 0.0002 to 9.74. In five of the six patients it was possible to observe a trend in the *BCR-ABL/ABL* ratio which corresponded with clinical course and treatment (Figure 5.7a, b, d-f). In four patients where samples were available prior to relapse, high *BCR-ABL/ABL* ratios (>0.01) and/or an apparent increase of the *BCR-ABL/ABL* ratio were seen several months before relapse, although the extent of the increase inevitably depended on the timing of the samples (Figure 5.7a, b, d, f). Five patients achieved clinical remission after DLI and this was reflected in a subsequent decline of the *BCR-ABL/ABL* ratio in samples available from 4 of these patients (Figure 5.7a, b, d, f). One patient remained in chronic phase post-relapse, and no decrease in the *BCR-ABL/ABL* ratio in this patient had been observed by the time of the last available sample at 4 months after the third course of DLI (Figure 5.7e). In one patient, the only sample of the 5 analyzed which had a *BCR-ABL/ABL* ratio >0.001 was when the patient was in clinical remission, although the patient had relapsed about 3 months earlier (Figure 5.7c).

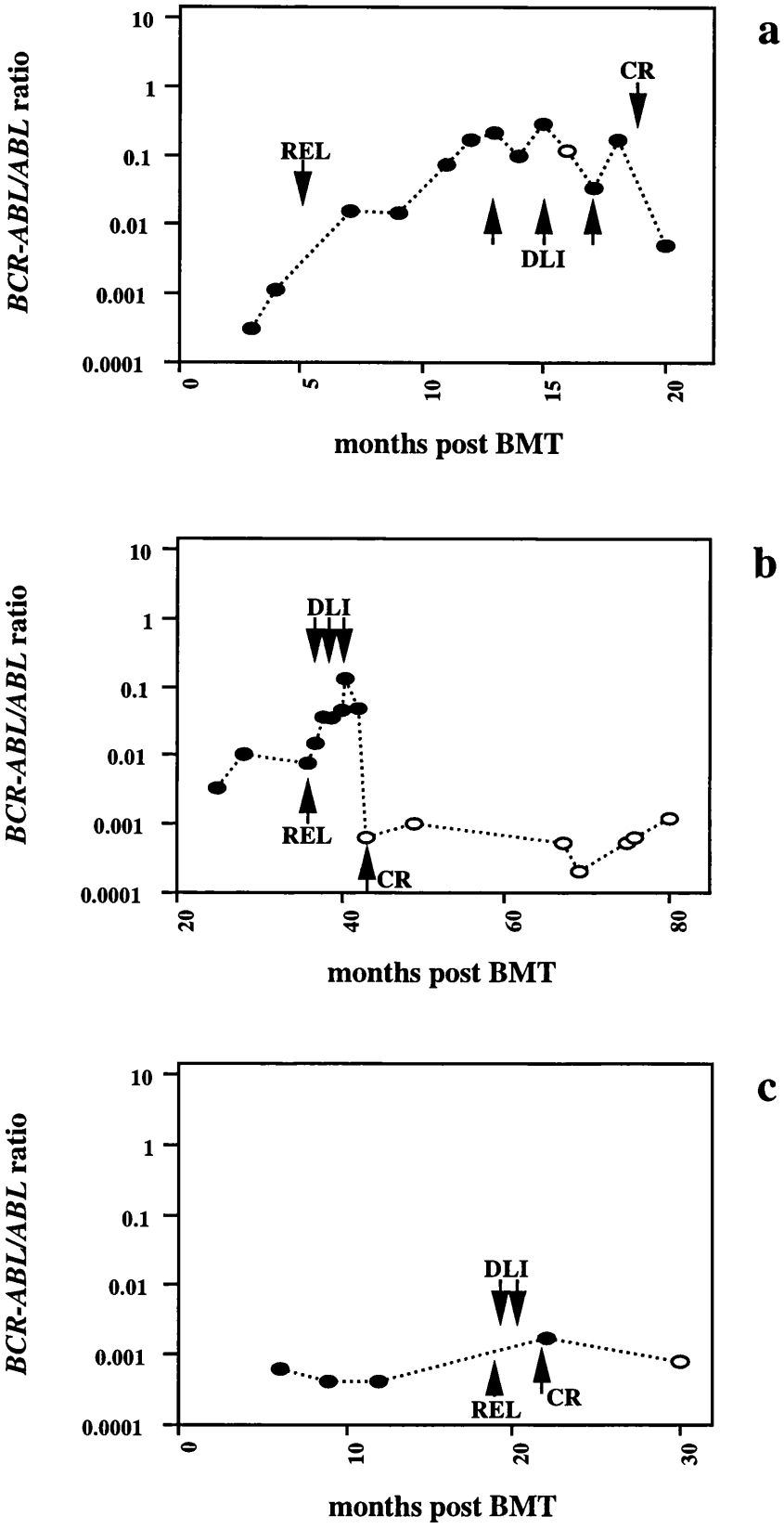
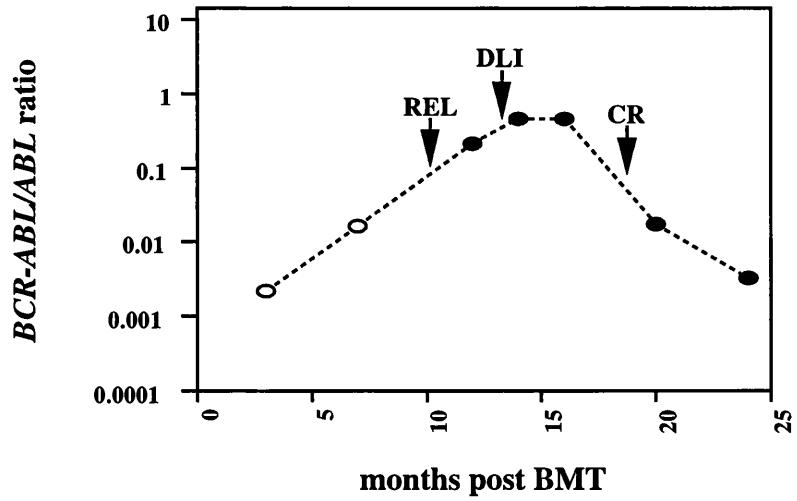
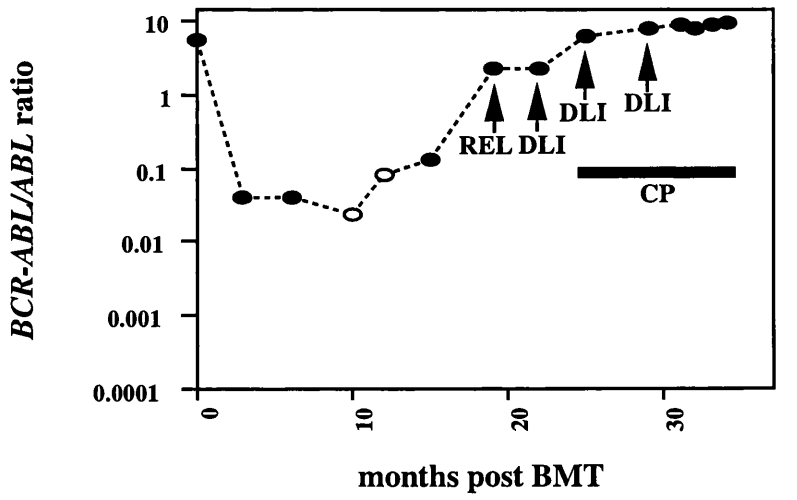
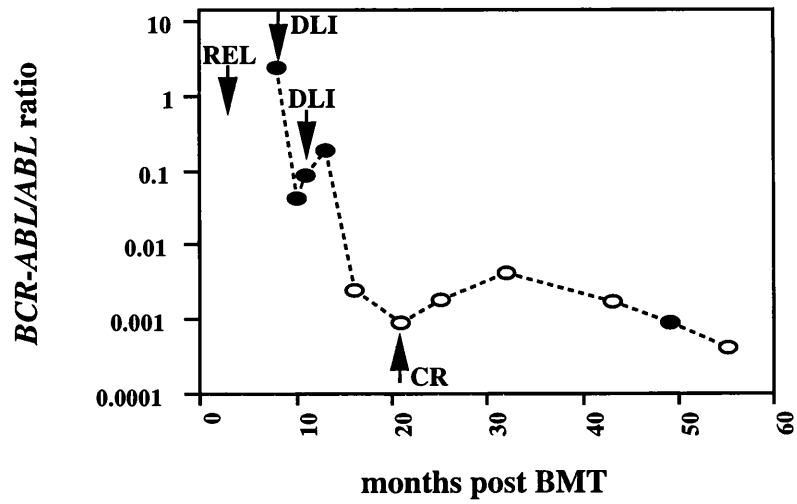


Figure 5.7 Sequential analysis of 6 patients who underwent alloBMT and subsequently relapsed at the molecular (**a-c**) or cytogenetic/haematological level (**d-f**). CR: complete remission; REL: relapse; DLI: donor lymphocyte infusion; CP: chronic phase; ●: RT-PCR positive; O: RT-PCR negative.


d
BCR-ABL/ABL ratio
10
1
0.1
0.01
0.001
0.0001
REL
DLI
CR
0
5
10
15
20
25
months post BMT


e
BCR-ABL/ABL ratio
10
1
0.1
0.01
0.001
0.0001
REL
DLI
DLI
DLI
CP
0
10
20
30
months post BMT


f
BCR-ABL/ABL ratio
10
1
0.1
0.01
0.001
0.0001
REL
DLI
DLI
CR
0
10
20
30
40
50
60
months post BMT

ABL levels for all samples from this patient were within the accepted range (8.5 – 73.3 ng), indicating that this result was not due RNA degradation.

When the TMA/HPA results were compared with conventional RT-PCR analysis, there was no consistent *BCR-ABL/ABL* ratio which separated results which were PCR positive or negative. However, 35 out of 39 samples analysed with a TMA/HPA *BCR-ABL/ABL* ratio >0.01 were positive by PCR analysis; conversely, 15 out of 27 PCR-negative samples had ratios <0.01.

5.5 Discussion

Although there were hopes that qualitative RT-PCR of the *BCR-ABL* fusion transcript in CML might be useful as an indicator of MRD, studies have shown that PCR positivity may persist after alloBMT, and in general this method is an unreliable predictor of impending relapse. As a consequence, a number of quantitative PCR methods have been introduced, but they either require multiple analyses of a single sample or expensive, sophisticated equipment. An alternative method, TMA/HPA, was therefore evaluated for quantifying the level of *BCR-ABL* and *ABL* transcripts in RNA samples from patients and controls. It differs in many aspects from PCR. Whereas the latter involves one enzyme activity and a maximum of a 2-fold increase in product at every discrete cycle, there are 2 very separate activities simultaneously taking place in TMA: (1) an RNA polymerase activity (comparable to a typical *in vitro* transcription reaction) and (2) a DNA polymerase function performed by the reverse transcriptase (comparable to the initial RT step in RT-PCR). Many transcripts or amplicons are made by RNA polymerase from each molecule of starting template, and each of these amplicons is immediately converted into a new template molecule for RNA polymerase by the RT activity. Since each of the created double stranded DNA template molecules can lead to the production of 100-1,000 RNA amplicons, this expansion can result in billions of amplicons produced within one hour and the fold increase is therefore

considerably greater than in PCR. The amplicons are then quantified in the HPA by hybridising to chemiluminescently-labelled sequence-specific probes.

Both component parts of this assay have been described previously but separately. The technique nucleic acid sequence based amplification (NASBA) is fundamentally very similar to TMA but uses MMLV-RT instead of AMV-RT (Compton, 1991). These enzymes have different specificities and efficiencies of incorporation, and NASBA also requires a supplementary addition of RNAse H. It has been successfully used to amplify *BCR-ABL* transcripts in a small number of diagnostic CML samples where the products were detected using ethidium bromide (Sooknanan *et al*, 1993). HPA has been reported as a rapid, sensitive, non-radioactive alternative to hybridisation of radioactively-labelled probes to semi-quantify *BCR-ABL* transcripts following conventional RT-PCR (Dhingra *et al*, 1991).

In the present study TMA and HPA have been combined to amplify, detect and quantify *BCR-ABL* and *ABL* transcripts. The results have been converted into an 'absolute' value in nanograms of post-amplification product by equating the chemiluminescent signals from the amplicons produced using 100 ng patient RNA with the signals from known amounts of K562 RNA assayed at the same time. Levels of *ABL* transcripts were used as an indicator of RNA integrity and only samples with an *ABL* result >0.5 ng, the background level for *ABL* detection, were accepted. In order to account for variations in RNA quality, results were normalised by expressing them as *BCR-ABL/ABL* ratios.

Calibration curves using varying amounts of K562 RNA demonstrated that linear results could be obtained over a range of 0.05 - 50 ng starting K562 RNA for *BCR-ABL* transcripts (Figure 5.4a) and 0.5 - 500 ng for *ABL* transcripts (Figure 5.4b). It is likely that linearity was lost above a certain copy number, 50 ng K562 RNA in the case of *BCR-ABL*, because the amplification machinery became saturated. A time course of the TMA incubation at 42°C indicated that most/all of the reaction occurred within the first 60 minutes and then slowed down, creating a stable plateau value (Figure 5.5a). One hour was therefore chosen as a

suitable time to measure the amount of product. The results obtained were reproducible, as shown by repeated simultaneous analysis of 3 different RNA samples from patients with varying levels of either *BCR-ABL* or *ABL* transcripts (Figure 5.5b), and by separate analysis of 4 different dilutions of K562 RNA (Figure 5.5c).

BCR-ABL levels were undetectable or at background level only (0.005 ng) in RNA from all normal controls. They were high (0.2 - 43.0 ng) in RNA from PB or BM of 18 patients at diagnosis who had evidence of *BCR-ABL* positivity by conventional RT-PCR. The normalised *BCR-ABL/ABL* ratios for these 18 patients were all >0.1. Only one patient had a *BCR-ABL* value which was slightly greater than that obtained using 50 ng of K562 RNA, the highest template concentration using in the regression analysis. Although dilution of the RNA and re-analysis would have provided a more accurate *BCR-ABL* value for this patient, this would not have altered interpretation of the result. Samples from two t(9;22) CML patients with very low or undetectable levels of *BCR-ABL* were also *BCR-ABL* negative by conventional RT-PCR and it is probable that these patients had *BCR* breakpoints outside the M-BCR (Melo, 1997).

In five out of six patients studied who relapsed following alloBMT and received DLI treatment, results demonstrated changes in *BCR-ABL* levels and *BCR-ABL/ABL* ratios which reflected patient status or treatment. Apparent increases in levels were detected several months before relapse and were more discriminatory than conventional RT-PCR, which was consistently positive throughout this period in some cases (Figure 5.7a, b). Similarly, levels decreased following DLI treatment, despite persistent PCR positivity in some patients (Figure 5.7a, d, e). Furthermore, although sequential analysis of individual patients was the most informative means of assessing their clinical status, a *BCR-ABL/ABL* ratio of 0.01 served as a useful cut-off point. Of the 18 remission samples post-DLI treatment analysed from these five patients, 17 had values below this level, and the remaining sample had a ratio of 0.017 (Figure 5.7d). Conversely, all samples with values ≥0.01 were associated with relapse. Results from the sixth patient were considered to be uninterpretable as they were all

<0.01 (Figure 5.7c). The reason for this failure is not clear as *ABL* levels on all samples were within the accepted range. However, it is possible that the crucial events have been missed as no samples were available for analysis between 12 and 22 months post alloBMT, including at the time of relapse (19 months) and DLI treatment (approximately 20 months).

TMA/HPA offers several advantages over conventional qualitative and quantitative RT-PCR techniques. It is rapid, with a total assay time of less than 4 hours, and requires only small amounts of RNA, less than 500 ng per triplicate analysis. The sensitivity is approximately equivalent to that of 'real-time' PCR, i.e. detecting one K562 cell in 10^4 – 10^5 HL60 cells, but it does not require expensive thermocycling equipment, simply two waterbaths to provide the different temperatures used for the amplification and hybridisation steps, and a basic luminometer. The whole procedure is carried out in one tube which minimises the risk of cross-contamination, and double-stranded DNA will not interfere with the assay as it will not act as a template for the initial reverse transcription step. Furthermore, the RNA amplicons produced in the TMA reaction are considerably more labile than DNA PCR products, substantially reducing the risk of carryover contamination and false positives. With appropriate primers and probes, TMA/HPA can be readily adapted to other translocations using the same core reagents.

Chapter 6

TMA/HPA analysis of *AML1-ETO* in AML

6.1 Introduction

The balanced t(8;21) chromosomal abnormality is most commonly seen in patients with AML of FAB type M2 but has also been reported in other FAB subtypes (Mitelman, 1995). Detection of this rearrangement is not only important in the correct assignment of disease subtype allowing appropriate and optimal treatments, but also provides a marker with which it is possible to monitor the patient throughout the clinical course of their disease. This monitoring may provide useful information of a patients' response to therapy and may also be of value in heralding an impending relapse, thus allowing clinical intervention before apparent haematological or clinical indications. Cases of AML with the t(8;21) are deemed to have a relatively superior overall survival in response to intensive chemotherapy, and employment of BMT in these patients has been shown to confer no significant survival advantage (Burnett *et al*, 1998; Bloomfield *et al*, 1998).

The t(8;21) can be demonstrated by conventional cytogenetic techniques, but as only a limited number of metaphases are analysed routinely, karyotypic analysis is a relatively insensitive means of monitoring MRD. As the resulting *AML1-ETO* fusion gene has been shown to be transcriptionally active in myeloid cell lineages and also in a fraction of CD34+ cells, monocytes an B lymphocytes (Miyamoto *et al,* 2000), fusion specific transcripts may be detected by RT-PCR (Kozu *et al*, 1993) (see Chapter 4). With the increasing use of RT-PCR, it has also become apparent that in a number of cases of AML, there is molecular evidence of the fusion gene without the corresponding cytogenetic abnormality (Maruyama *et al*, 1993; Andrieu *et al*, 1996; Krauter *et al*, 1998; Mitterbauer *et al*, 1998; Sarriera *et al*, 2001). However, the sensitivity of qualitative RT-PCR techniques varies between detection of one *AML1-ETO* positive cell in 10^4 - 10^6 *AML1-ETO* negative cells depending on the number of rounds of PCR or use of a confirmatory hybridisation step (Satake *et al*, 1995;

Morschhauser *et al*, 2000). Many patients remain *AML1-ETO* PCR positive in long term remission. Kusec *et al* (1994) demonstrated RT-PCR positivity, at a detection level of one *AML1-ETO* expressing cell in 10^5 non-leukaemic cells, in patients in CR for over 6 years following chemotherapy and in patients in CR for over one year following autoBMT whereas Jurlander *et al* (1996) showed PCR positivity in patients in CR for over 6 years following alloBMT using an assay with a similar sensitivity. The usefulness of these types of studies in predicting clinical outcome therefore remains controversial (Preudhomme *et al*, 1996).

As with other translocation generated fusion transcripts, methods have been introduced to quantify *AML1-ETO* transcripts, including competitive and 'real-time' RT-PCR. *AML1-ETO* competitor molecules can be constructed which contain the primer sequences necessary for *AML1-ETO* PCR but which produce a fragment of slightly larger size than the t(8;21) *AML1-ETO* transcript. Increasing amounts of competitor are added to a series of PCR reactions from patient cDNA and comparison of the resulting product intensities is used to estimate the level of transcript in the patient sample. This method has been shown to be valuable in predicting early relapse (Tobal & Liu Yin, 1996), identifying patients in durable remission (Tobal *et al*, 2000) and also in evaluating *AML1-ETO* positive cell contamination in peripheral blood stem cell harvests of t(8;21) AML patients (Miyamoto *et al*, 1995). However, this method requires multiple PCR reactions for each sample, which is time consuming, and reproducibility is variable (Freeman *et al*, 1999). 'Real-time' PCR estimates the number of *AML1-ETO* molecules on the basis of the number of amplification cycles required to reach a threshold level of detection (Haferlach, 2001). Studies using 'real-time' quantification have been used to demonstrate a reduction in *AML1-ETO* levels during induction chemotherapy (Fujimaki *et al*, 2000; Kondo *et al*, 2000; Wattjes *et al*, 2000) and increasing levels before haematological relapse (Marcucci *et al*, 1998; Krauter *et al*, 1999). The sensitivity of this methodology is similar to that of

competitive RT-PCR and can detect one *AML1-ETO* positive Kasumi-1 cell in the presence of 10^4 - 10^5 *AML1-ETO* negative cells but, as discussed in Chapter 5, it requires sophisticated and expensive equipment.

The RNA-based TMA and HPA assay described in Chapter 5 was therefore evaluated as a means of quantifying *AML1-ETO* transcripts in peripheral blood or bone marrow samples from t(8;21) AML patients.

6.2 Materials and Methods

6.2.1 Patients

RNA was analysed from 20 haematologically normal controls (HNCs) (BM n=10, PB n=10), from 20 AML patients at diagnosis with a classical t(8;21) translocation (BM=10, PB=10), from 3 AML patients at diagnosis with three-way variant t(8;V;21) translocations (BM n=2, PB n=1) and from 17 AML patients at diagnosis who were *AML1-ETO* RT-PCR positive without cytogenetic evidence of t(8;21) [t(8;21)-*/AML1-ETO*+] (BM n=11, PB n=6) as described in Chapter 4. Sequential samples from a further 7 t(8;21) AML patients taken throughout treatment course were also analysed.

6.2.2 Sample preparation

RNA was extracted as described in Chapter 3.2.3

6.2.3 RT-PCR analysis

RT-PCR analysis for *AML1-ETO* transcripts was performed as described in Chapter 4.2.4 and for *ABL* transcripts as described in Chapter 3.2.4.

6.2.4 TMA/HPA analysis

The methodology of TMA and HPA are described in Chapter 5.3.5 and 5.3.6 respectively. Five hundred ng of RNA was used per test in the *AML1-ETO* assay whereas 100 ng was used in the *ABL* assay as described in Chapter 5.3.5. Fifteen pmol each of promoter-primer (Table 5.1 for *ABL* promoter primer) and reverse primer Table 5.1 for *ABL* reverse primer) were used per test. One hundred microlitres of probe mix containing 7.5 nM AE-labelled oligonucleotide probe complementary either to the *AML1-ETO* product or to an *ABL* exon-1b sequence (Table 5.1) were used in the relevant HPA. TMA primers and HPA probes were manufactured and supplied by Gen-Probe Incorporated, San Diego, CA, USA. Sequences of the *AML1-ETO* promoter primer, reverse primer and AE-labelled probe are not disclosed as they are currently covered by USA patent review regulations.

6.2.5 Sensitivity

In order to evaluate the sensitivity of the technique and linearity of the increase in RLUs obtained with increasing template concentration, the *AML1-ETO* and *ABL* signals from varying amounts (0 – 500 ng) of total RNA from the *AML1-ETO* positive cell line Kasumi-1 (Asou *et al*, 1991) were measured as described above. In addition, RNA was extracted from mixtures containing 1 Kasumi-1 cell in 10, 10^2, 10^3, 10^4, 10^5 or 10^6 HL60 cells (*AML1-ETO* negative): 100 ng and 500 ng of this RNA was used to assay the *ABL* and *AML1-ETO* signals respectively.

6.2.6 Reproducibility

Sample reproducibility was assessed using RNA from 3 patients with high, medium and low *AML1-ETO* levels which were selected from preliminary analysis of t(8;21) AML patients at diagnosis. Eight separate aliquots were prepared from each sample, each of 1.5 μg, and assayed in triplicate (500 ng/test) using the same batch of reagents.

6.2.7 Statistical analysis

Simple regression analysis was used to verify the linearity of calibration curves. Student's t-test (unpaired, two tailed, equal variance) was employed to analyse the difference in both *AML1-ETO* and *ABL* levels of t(8;21), *AML1-ETO* and control patient samples: a p value of less than 0.05 was deemed to be significant. CVs were calculated to assess technical variation in multiple sample analysis.

6.3 Results

6.3.1 Technical evaluation of TMA/HPA

6.3.1.1 Sensitivity and linearity

In the *AML1-ETO* assay, levels above background were detected using greater than 0.05 ng Kasumi-1 RNA (Figure 6.1a) and this correlated to the detection of 1 Kasumi-1 cell in 10^3 - 10^4 HL60 cells (Figure 6.1c). Regression analysis of the RLU values obtained for 0.05, 0.5, 5 and 50 ng starting RNA gave r^2 values of 0.910 - 0.999 (mean 0.976, median 0.984) in 6 analyses. The *ABL* assay required greater than 0.5 ng Kasumi-1 RNA to detect levels above background (Figure 6.1b). Regression

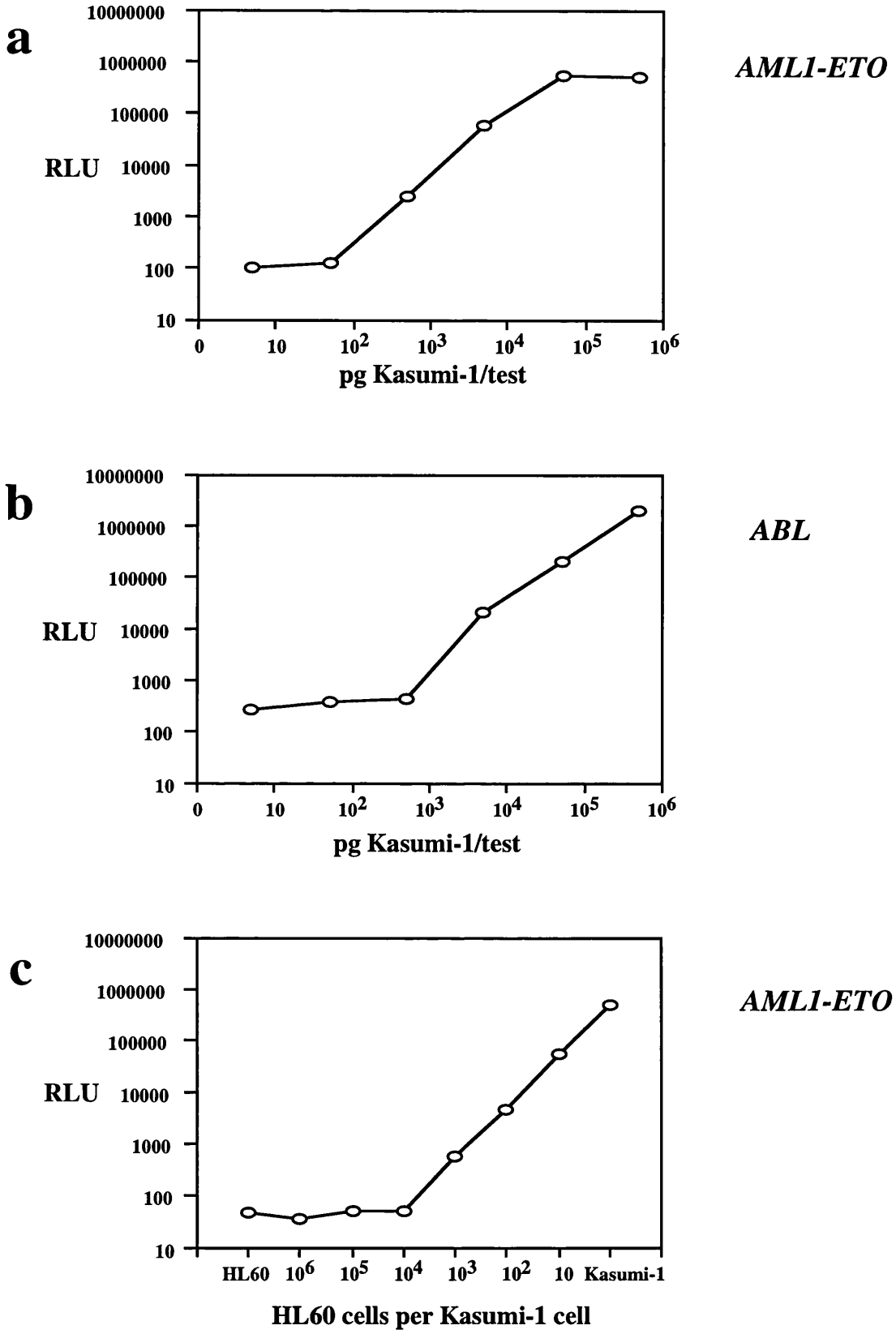


Figure 6.1 Linearity and sensitivity of assays. Increasing concentrations of Kasumi-1 RNA were assayed for levels of (**a**) *AML1-ETO* and (**b**) *ABL* transcripts. (**c**) *AML1-ETO* levels were assayed in RNA from one Kasumi-1 cell in the presence of increasing numbers of HL60 cells.

analysis of the *ABL* values obtained for 0.5, 5, 50 and 500 ng starting RNA gave r^2 values of 0.953 - 0.997 (mean 0.976, median 0.972) in 6 analyses.

6.3.1.2 Reproducibility

The results obtained for assessment of reproducibility are shown in Figure 6.2a. The CVs of the mean values from the 8 aliquots were 12.4%, 8.5% and 11.0% for the 3 samples respectively. Day to day technical variation was assessed using the data from the 6 calibration curves created using 4 different dilutions of Kasumi-1 RNA (Figure 6.2b). The CVs were 19.6%, 21.8%, 32.8% and 25.9% for 50, 5, 0.5 and 0.05 ng RNA respectively in the *AML1-ETO* assay.

6.3.2 Analysis of AML patient samples

For each series of patient samples analysed, calibration curves using samples of 0.05, 0.5, 5 and 50 ng starting Kasumi-1 RNA for *AML1-ETO* analysis and 0.5, 5, 50 and 500 ng for *ABL* analysis were constructed as described above and the slopes used to convert RLUs obtained into post-amplification RNA quantities. Five hundred and 100 ng template RNA was selected as the optimal amount to use per test in the *AML1-ETO* and *ABL* assays respectively as this gave post-amplification levels within the linear range of the curve for samples with varying levels of target.

6.3.2.1 Evaluation of RNA integrity

By plotting paired *ABL* and *AML1-ETO* levels of individual samples it was possible to graphically identify those patient samples with poor RNA quality (Figure 6.3). Seven samples with an *ABL* level <0.5 ng were subsequently excluded from further analysis. One was from an HNC (Figure 6.4b), one from a t(8;21)/t(8;V;21) patient (Figure

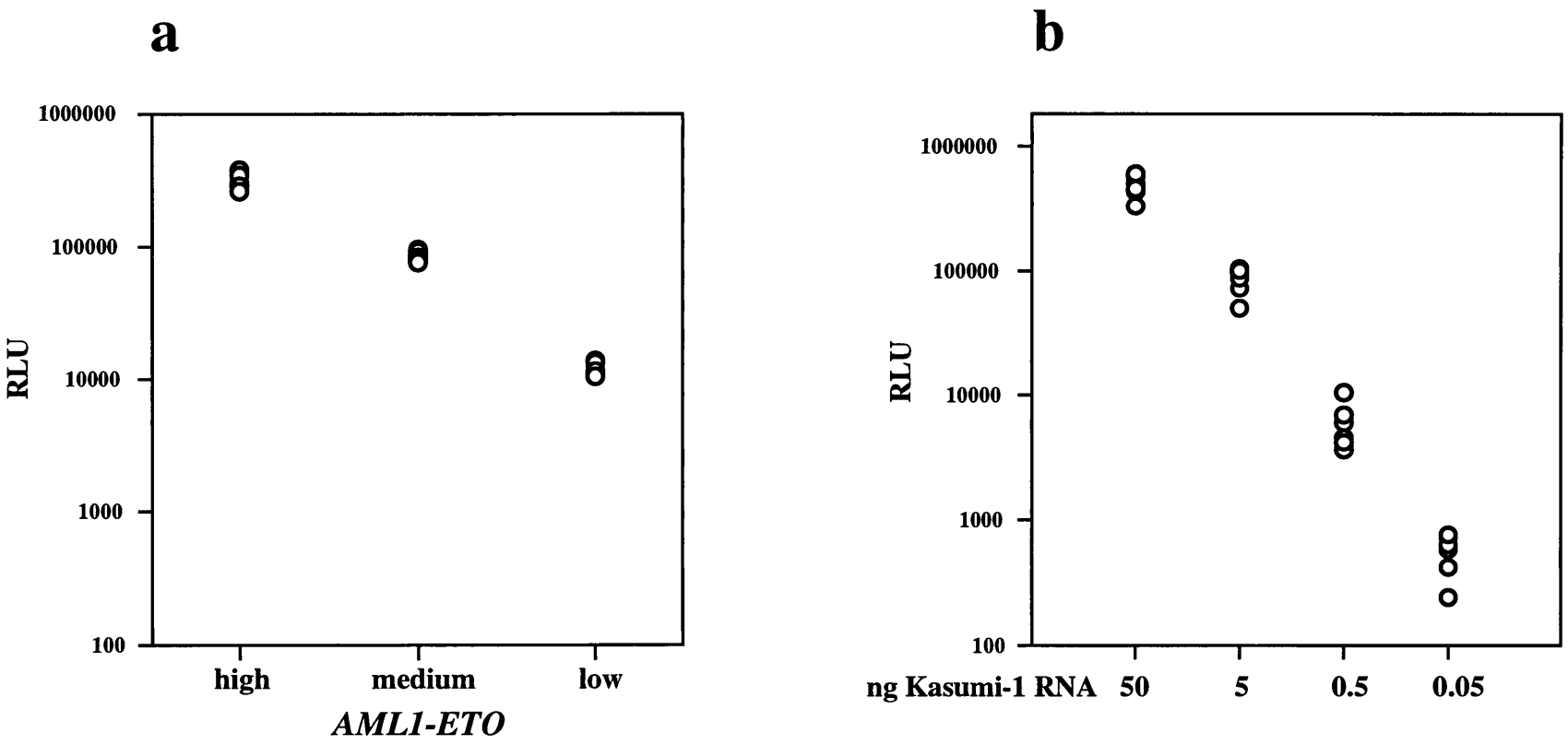


Figure 6.2 Reproducibility of *AML1-ETO* TMA/HPA. (**a**) Repeated analysis of samples with varying *AML1-ETO* levels. Each sample was assayed 8 times in triplicate and (**b**) day to day variation of 4 different concentrations of Kasumi-1 RNA assayed on 6 separate occasions.

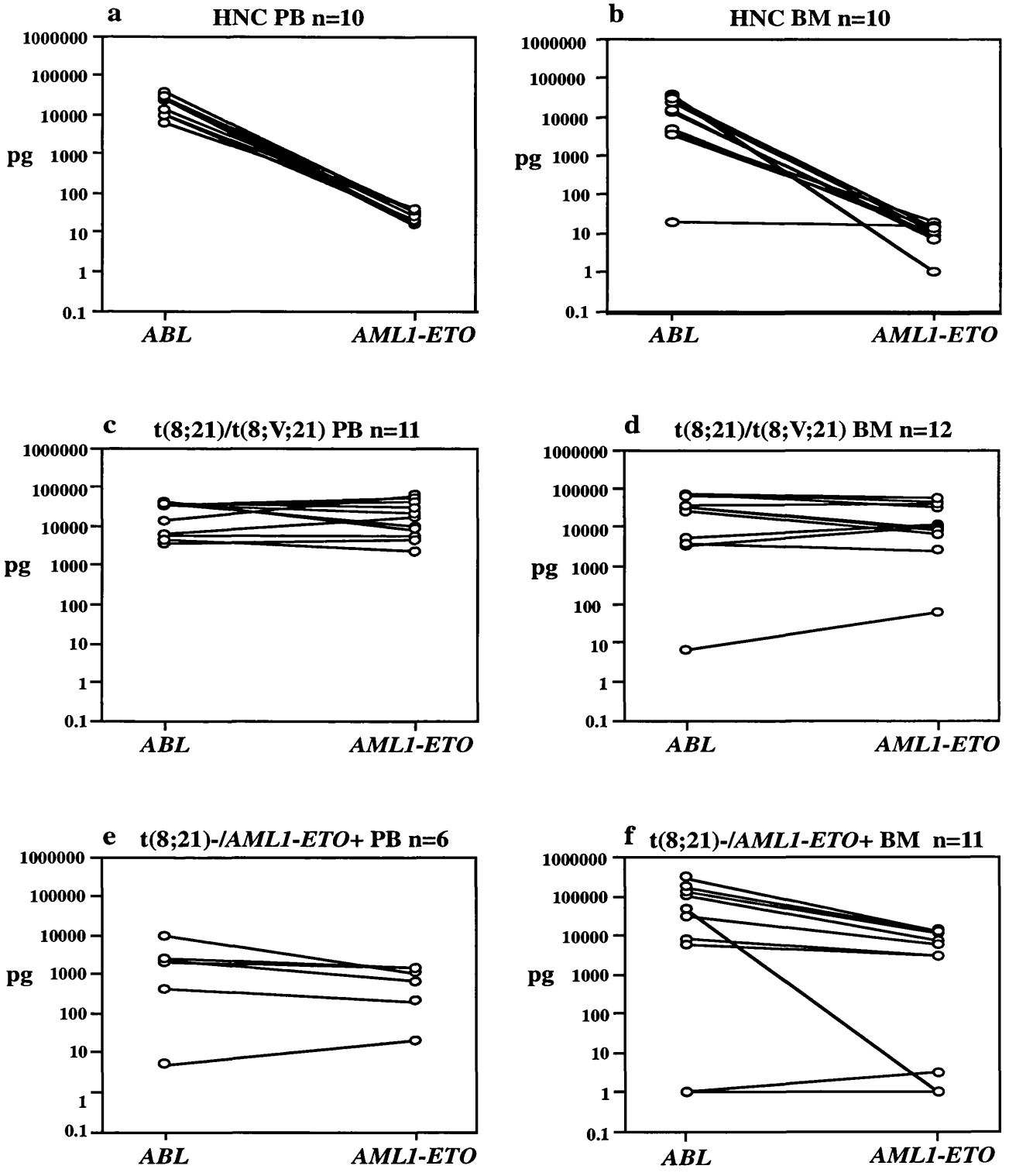


Figure 6.3 Evaluation of RNA integrity by plotting paired *AML1-ETO* and *ABL* levels for individual samples analysed. Samples with no *ABL* expression (**b, d-f**) were excluded from further analysis. One t(8;21)-/*AML1-ETO*+ patient is shown to have a possible false-positive RT-PCR result (**f**).

6.4d) and five from t(8;21)-/*AML1-ETO*+ patients (Figure 6.4e,f). This representation also demonstrated that one sample appeared to be false positive for *AML1-ETO* by RT-PCR (Figure 6.3f).

6.3.2.2 Diagnosis

Nineteen HNC samples (9 BM, 10 PB) had a median *ABL* level of 22.3 ng (range 3.5 - 37.4) (Figure 6.4b), *AML1-ETO* levels which were not above background (0.05 ng) (Figure 6.4a) and *AML1-ETO/ABL* ratios less than 0.01 (Figure 6.4c). *ABL* levels were above background level (range 3.5 - 67.8 ng, median 32.7 ng) in 22 t(8;21)/t(8;V;21) patients (12 BM, 10 PB), *AML1-ETO* levels ranged between 2.3 and 64.5 ng (median 14.7 ng) and all had *AML1-ETO/ABL* ratios > 0.1 (median 0.815, range 0.1951 - 4.968) (Figure 6.4c). The 12 t(8;21)-/*AML1-ETO*+ patients (8 BM, 4 PB) had *ABL* levels between 2.2 and 321.5 ng (median 9.7 ng) (Figure 6.4b). Eleven of these 12 had *AML1-ETO* levels above background (range 0.7 - 13.9 ng, median 3.0 ng) (Figure 6.4a) and *AML1-ETO/ABL* ratios ranging from 0.043 - 0.630 (median 0.202) (Figure 6.4c). The remaining sample, originally classified as t(8;21)-/*AML1-ETO*+, had an *ABL* level of 47.6 ng and no detectable *AML1-ETO* transcripts. Repeat *AML1-ETO* RT-PCR analysis for this sample was negative suggesting a previous false positive result. There was no significant difference between the *ABL* levels of the AML and HNC samples (p=0.1). The *AML1-ETO* levels were significantly higher in the 33 t(8;21)/t(8;V;21) and t(8;21)-/*AML1-ETO*+ patients than the HNCs (p=0.001). Also of note is that the *AML1-ETO* levels and *AML1-ETO/ABL* ratios of the 22 t(8;21)/t(8;V;21) AML patients were significantly higher than the 11 t(8;21)-/*AML1-ETO*+ patients (p=0.016).

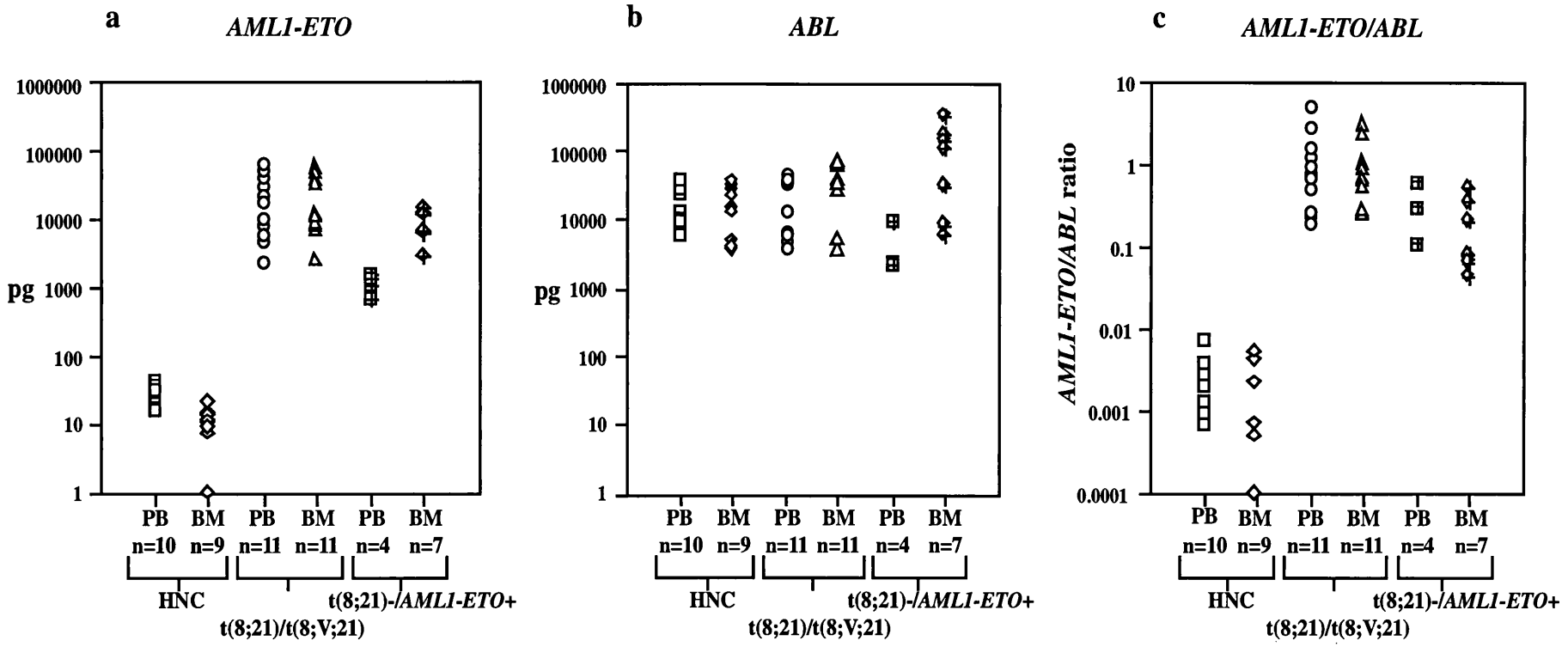


Figure 6.4 Post-amplification levels of (**a**) *AML1-ETO* and (**b**) *ABL* using RNA from 19 HNCs, 22 t(8;21)/t(8;V;21) AML patients and 11 t(8;21)-/*AML1-ETO*+ patients at diagnosis. (**c**) The corresponding *AML1-ETO/ABL* ratios.

6.3.2.3 During treatment

Sequential levels of *AML1-ETO* and *ABL* transcripts were quantified in 500 ng and 100 ng total RNA respectively from PB or BM of seven t(8;21) AML patients either from diagnosis or first relapse. One patient remained in CR (Figure 6.5c) whereas the other 6 patients all relapsed. *ABL* levels ranged from 1.6 – 97.8 ng (median 16.7 ng), which was similar to that in the control and diagnostic samples. *AML1-ETO/ABL* ratios ranged from less than 0.0001 to 8.04. In all 7 patients it was possible to observe a trend in the *AML1-ETO/ABL* ratio which corresponded with clinical course and treatment (Figure 6.5a-g). In the 3 patients where samples were available between attainment of CR and relapse, an increase of the *AML1-ETO/ABL* ratio was observed 1 - 4 months prior to relapse (Figure 6.5a, e, f) which was reflected by at least a 2 log increase in the *AML1-ETO/ABL* ratio. All patients achieved either CR1 or CR2 and this was reflected in a decline of the *AML1-ETO/ABL* ratio in samples available from all patients. One patient remained in CR, and no increase in the *AML1-ETO/ABL* ratio in this patient was observed (Figure 6.5c).

When the TMA/HPA results were compared with conventional RT-PCR analysis, there was a consistent *AML1-ETO/ABL* ratio which separated results which were PCR positive or negative. All 13 samples analysed with a TMA/HPA *AML1-ETO/ABL* ratio >0.1 were positive by PCR analysis; conversely, 25 out of 26 PCR-negative samples had ratios <0.1 (Figure 6.5).

6.4 Discussion

As qualitative RT-PCR techniques to detect *AML1-ETO* fusion transcripts in t(8;21) AML were developed, it was thought that these tests would be a useful indicator of MRD. Studies have since shown that PCR positivity may persist in long term

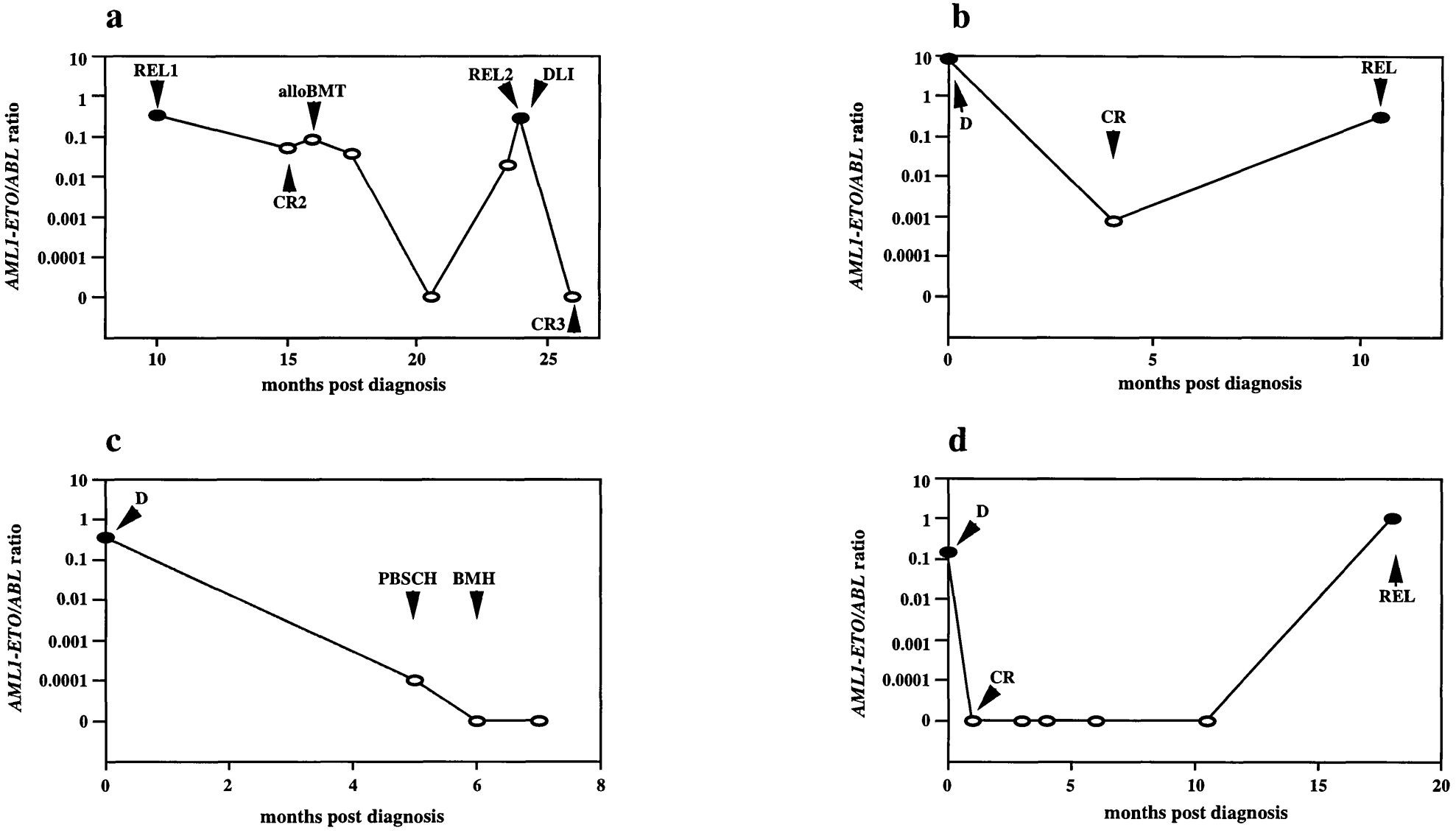
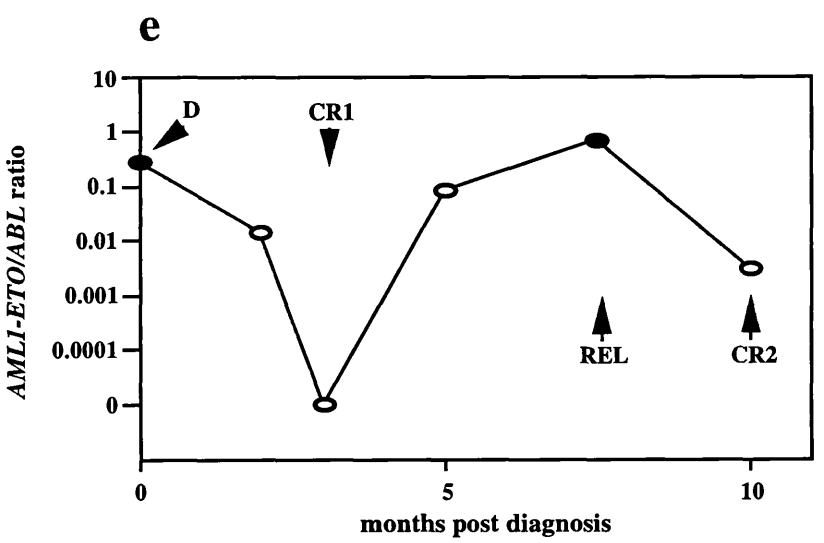
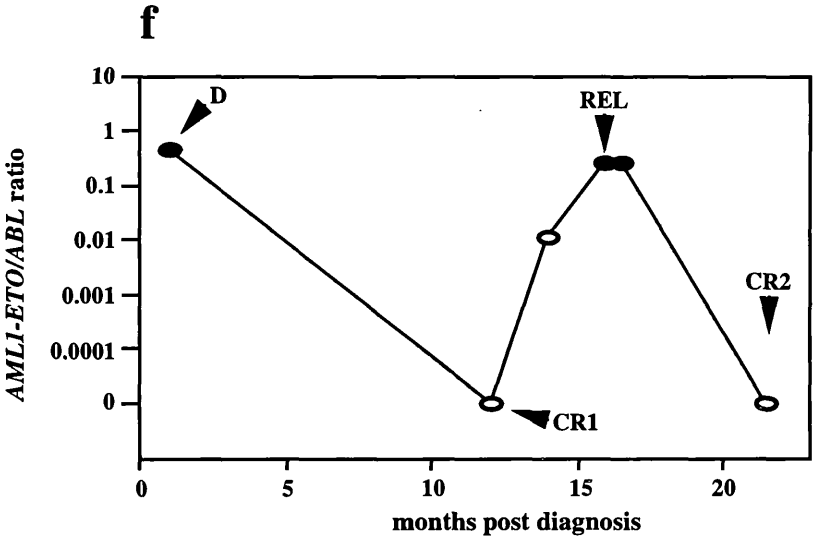
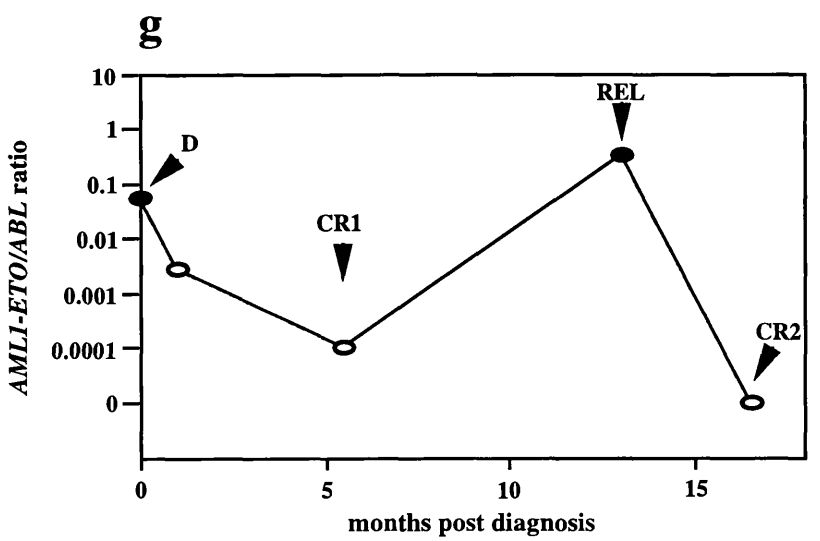


Figure 6.5 Sequential analysis of 7 t(8;21) AML patients. D: diagnosis; CR: complete remission; REL: relapse; DLI: donor lymphocyte infusion; PBSCH: peripheral blood stem cell harvest; BMH: bone marrow harvest; ●: RT-PCR positive; O: RT-PCR negative.


e
AML1-ETO/ABL ratio
10
1
0.1
0.01
0.001
0.0001
0
D
CR1
REL
CR2
0
5
10
months post diagnosis


f
AML1-ETO/ABL ratio
10
1
0.1
0.01
0.001
0.0001
0
D
REL
CR2
CR1
0
5
10
15
20
months post diagnosis


g
AML1-ETO/ABL ratio
10
1
0.1
0.01
0.001
0.0001
0
D
CR1
REL
CR2
0
5
10
15
months post diagnosis

remission, even after alloBMT, and therefore this method is unreliable for predicting impending relapse. Quantitative PCR methods have been introduced, but as discussed previously, they either require multiple analyses of a single sample or expensive, sophisticated equipment (Wattjes *et al*, 2000). An alternative method of TMA/HPA for quantifying the level of control *ABL* and AML associated *AML1-ETO* transcripts in RNA samples from patients and controls was therefore evaluated. The results have been converted into an 'absolute' value in nanograms of post-amplification product by equating the chemiluminescent signals from the amplicons produced using 500 ng patient RNA with the signals from known amounts of *AML1-ETO*+ Kasumi-1 RNA assayed at the same time. Calibration curves using varying amounts of Kasumi-1 RNA demonstrated that linear results could be obtained over a range of 0.05 - 50 ng starting Kasumi-1 RNA for *AML1-ETO* transcripts (Figure 6.1a) and 0.5 - 500 ng for *ABL* transcripts (Figure 6.1b). The results obtained were reproducible, as shown by repeated simultaneous analysis of 3 different RNA samples from patients with varying levels of *AML1-ETO* transcripts (Figure 6.2a), and by separate analysis of 4 different dilutions of Kasumi-1 RNA (Figure 6.2b).

Levels of *ABL* transcripts were used as an indicator of RNA integrity and only samples with an *ABL* result >0.5 ng, the background level for *ABL* detection, were accepted. Failure of *ABL* amplification in one sample from a t(8;21) AML patient, one sample from an HNC and 5 samples from t(8;21)-/*AML1-ETO*+ patients at diagnosis possibly reflects the effect of multiple freeze-thaw cycles undergone by these RNA samples. *ABL* was selected as a control for this quantitative assay as it is stably expressed in myeloid cells at a level similar to *AML1-ETO* and other translocation associated fusion transcripts (Lion, 2001). This stable expression of *ABL* is apparent when comparing levels from known dilutions of K562 and Kasumi-1 RNA. In the creation of calibration curves, median *ABL* RLUs were 21,990, 176,374 and 2,577,971 using 5, 50 and 500 ng of K562 RNA respectively (Figure 5.4b) and

20,764, 196,195 and 2,033,639 using the same amounts of Kasumi-1 RNA respectively (Figure 6.1b). Comparable *ABL* levels were also observed in patients irrespective of the disease under investigation: range 0.6 - 82.6 ng for t(9;22)+ CML patients and range 3.5 - 67.8 ng for t(8;21)/t(8;V;21)+ AML patients. In order to account for variations in RNA quality, results were normalised by expressing them as *AML1-ETO/ABL* ratios.

AML1-ETO levels were undetectable or at background level only (0.005 ng) in RNA from 19 HNCs. They were high (range 2.3 - 64.5 ng) in RNA from PB or BM of 22 t(8;21)/t(8;V;21) patients at diagnosis who also had evidence of *AML1-ETO* positivity by conventional RT-PCR. The normalised *AML1-ETO/ABL* ratios for these 22 patients were all > 0.1. None of the patients had an *AML1-ETO* level greater than that obtained using 50 ng of Kasumi-1 RNA, the highest template concentration used in the regression analysis. The median *AML1-ETO* level (3.0 ng) of the 11 t(8;21)-*/AML1-ETO*+ patients was approximately five times lower than that of the t(8;21)/t(8;V;21) patients (14.7 ng) and this was reflected in a smaller median *AML1-ETO/ABL* ratio (0.202 versus 0.815). Of the t(8;21)-*/AML1-ETO*+ samples, 4 (36%) were derived from PB which could account for the difference in levels as there is generally a lower percentage of blasts in the blood, an hence more contaminating non-leukaemic cells, than in the BM at presentation of AML. The median percentage of blasts of the remaining 7 BM samples was 59% (range 39% - 92%). Of the 22 t(8;21)/t(8;V;21) patient samples studied, 11 were from PB and in 3, the percentage BM blasts was unavailable. In the remaining 8 samples, the median % BM blasts was 85% (range 30% - 98%). If the t(8;21)-*/AML1-ETO*+ subset of patients have lower *AML1-ETO* levels at diagnosis, intensive treatment would result in levels below the detection limit of techniques using *AML1-ETO* transcripts as a marker of MRD, thus precluding early detection of molecular relapse.

In six of the seven patients studied sequentially and who relapsed (Figure 6.5a, b, d-g) a difference of at least 2 logs in the *AML1-ETO/ABL* ratio was observed between CR and subsequent relapse samples. In 3 of the patients studied (Figure 6.5a, e, f) an apparent increase in *AML1-ETO/ABL* ratio was detected before relapse and this was more discriminatory than conventional RT-PCR. Similarly, levels decreased following induction treatment in all 7 patients. Although sequential analysis of individual patients was the most informative means of assessing their clinical status, an *AML1-ETO/ABL* ratio of 0.1 served as a useful cut-off point. Of the 23 remission samples analysed from these 7 patients, all had values below this level. Conversely, all samples with values > 0.1 were associated with diagnosis or relapse. The remission samples in this study were *AML1-ETO* RT-PCR negative (Figure 6.5) which conflicts with previous studies suggesting that *AML1-ETO* transcripts are present in most t(8;21) AML patients in long-term remission (Kusec *et al*, 1994; Jurlander *et al*, 1995; Tobal *et al*, 2000). This apparent difference is probably due to a lower sensitivity of the RT-PCR assay employed (see Chapter 4), although whether increased sensitivity of qualitative assays provides any further clinical information of value remains controversial (Preudhomme *et al*, 1996; Haferlach, 2001).

This study emphasises the advantages TMA/HPA offers over quantitative RT-PCR techniques. TMA/HPA is rapid, requires only small amounts of RNA, does not require expensive thermocycling equipment and reduces the risk of carryover contamination and false positives. The sensitivity of the *AML1-ETO* assay is lower than that of 'real-time' PCR but can produce quantitative results which, in this small retrospective study, mirror clinical events. It has been shown that with appropriate primers and probes, the TMA/HPA format is flexible enough to be adapted to quantify other translocation generated fusion transcripts but requires some optimisation of input RNA quantity in order to achieve maximal amplification and detection.

Chapter 7

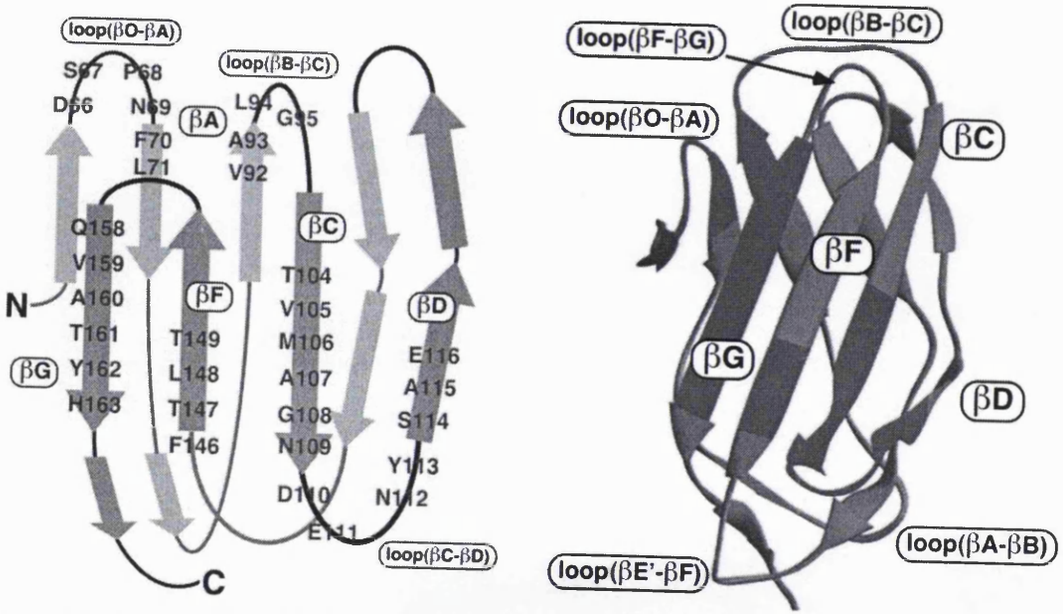
Mutational analysis of *AML1* in AML

7.1 Introduction

The *AML1* gene is a member of the runt family of genes which are involved in the transcriptional regulation of normal haemopoiesis and osteogenesis (Komori & Kishimoto, 1998; Lutterbach & Hiebert, 2000). *AML1*, located on chromosome 21, was first identified as the fusion partner to the *ETO* gene created by the t(8;21)(q22;q22) chromosomal translocation (Miyoshi *et al,* 1991) which occurs in approximately 10-15% of adult patients with AML. The sequence of a portion of *AML1* was found to be highly homologous to the *Drosophila* segmentation gene *runt* (Daga *et al,* 1992; Erickson *et al,* 1992) and to 2 other genes that encode proteins with DNA binding runt domains, *AML2* and *AML3* (Levanon *et al,* 1994). The *AML1* gene spans approximately 260Kb of DNA and contains 9 major exons (Miyoshi *et al,* 1995; Levanon *et al,* 2001). The runt domain is encoded by exons 3-5 and these 128 amino acids take up a folded conformation that allows AML1 binding to both DNA and CBFβ (Meyers *et al,* 1993). Although these interactions occur on different parts of the domain, binding of CBFβ substantially enhances the ability of AML1 to bind to DNA (Crute *et al,* 1996; Tang *et al,* 2000). The 3' sequence of *AML1* codes for a transactivation domain which is lost in the t(8;21) (Meyers *et al,* 1995). The predicted interaction surfaces identified in structural studies of runt domain complexes have been analysed by site-directed mutagenesis and have characterised the functional roles of specific amini acid residues of the AML1 molecule (Figure 7.1).

Evidence for the critical role of AML1 in haemopoiesis has come from *AML1* knock-out mice which are embryonic lethal and show a lack of foetal haemopoietic cells (Okuda *et al,* 1996; Wang *et al,* 1996). In particular, several lines of evidence indicate that AML1 plays an important role in myeloid differentiation. It activates a number of genes in immature myeloid cells such as myeloperoxidase and neutrophil elastase (Nuchprayoon *et al,* 1994) and interacts with the transcription factor C/EBPα which is

Figure 7.1 Predicted heterodimerisation surface of the runt domain.Left: Highlighted residues of the domain in contact with CBFB in a protein/DNA complex. Right: molecular structure of the runt domain in its DNA bound conformation. Dark shading indicates the segments in contact with CBFB whereas the βA-βB and βE'-βF loops form the DNA-binding surface. From Nagata & Werner (2001).



essential for granulocytic differentiation (Zhang *et al,* 1996 & 1997; Pabst *et al,* 2001a). In t(8;21) AML, the AML1-ETO protein lacks the transactivation domain, retains the runt domain, and acts in a dominant negative manner by competing with wild type alleles for the DNA binding site but without the ability to direct transcription of target genes (Meyers *et al,* 1995). Over-expression of AML1-ETO inhibits G-CSF induced differentiation of myeloid progenitor cells (Westendorf *et al,* 1998), which may be mediated through its association with and down-regulation of C/EBPα (Pabst *et al,* 2001b). Similarly, increased transcript levels of an alternatively spliced isoform (AML1a) which truncates the protein and deletes the transactivation domain have been reported in AML patients, and over-expression of AML1a dominantly suppresses transcriptional activation by the full length protein and inhibits granulocytic differentiation in response to G-CSF (Tanaka *et al,* 1995a).

Recent studies have identified point mutations, insertions and deletions in the *AML1* gene in a significant number of patients with AML and to a lesser extent in patients with MDS (Osato *et al,* 1999; Preudhomme *et al,* 2000; Imai *et al,* 2000). An initial report described *AML1* mutations in 6 AML patients, 3 of whom were from a group of 9 patients (33%) studied with the FAB type of minimally differentiated M0 AML (Osato *et al,* 1999). A further study found *AML1* mutations in 9/41 (22%) AML M0 patients (Preudhomme *et al,* 2000). Studies of haematological malignancies selected on the basis of the presence of trisomy 21 found *AML1* mutations in a case of M0 AML following essential thrombocythaemia (ET), a case of M1 AML following atypical CML and 2 other cases of M2 AML (Preudhomme *et al,* 2000). Overall, these 2 studies suggested that the incidence of *AML1* mutations in M0 AML is approximately 24% and in other forms of AML is less than 5%.

Interestingly, 5 of the 6 reported cases of non-M0 AML with *AML1* mutations had monoallelic disease whereas 11/12 evaluable cases of M0 AML had no wild type

AML1 gene (Osato *et al,* 1999; Preudhomme *et al,* 2000). It is thus conceivable that the lack of differentiation in a significant proportion of AML M0 patients is due to a severe deficiency of functional AML1 protein. The aim of this study was therefore to confirm the high incidence of mutations in the *AML1* gene in M0 AML and to determine whether this FAB type is associated with biallelic or monoallelic disease with loss of the wild type allele.

Germline mutations of *AML1* have also been demonstrated in a familial platelet disorder with a predisposition to AML (FPD/AML) (Song *et al,* 1999). This suggested a role for AML1 in megakaryopoiesis and raised the possibility that mutations of *AML1* could also contribute to AML in patients with FAB type M7 in which there is an excess of megakaryoblasts. To date, the studies by Osato *et al* (1999) and Preudhomme *et al* (2000) have only studied 2 such cases and therefore a larger number of patients with this rare AML type were analysed in this study.

7.2 Materials and methods

7.2.1 Patient samples

Bone marrow or peripheral blood was obtained from 61 adult patients with AML at diagnosis with either M0 (n=41) or M7 (n=20) according to FAB classification (Bennett *et al,* 1985 & 1991). Karyotype was available for 56 of the patients, none of whom had a t(8;21) and one of whom, a patient with M0, had trisomy 21. DNA was extracted from patient slides and samples as previously described (Fey *et al,* 1987; Gustincich *et al,* 1991) (see Chapter 2) and from the haemopoietic cell line TF-1 (Kitamura *et al,* 1989) which was used as a control.

7.2.2 PCR-SSCP analysis

For each patient, fragments encompassing exons 3, 4 and 5 were amplified using intronic PCR primers previously published by Song *et al* (1999) (Table 7.1). One microlitre of genomic DNA (100ng) was amplified in a 20µl PCR reaction with final concentrations of 0.5 units Taq DNA polymerase, 1 x Taq polymerase buffer (both Bioline, London, UK), 0.5 mM $MgCl_2$, 0.2 mM dNTPs and 200ng of each primer. Thirty five cycles were performed, each consisting of 95°C for 30 seconds, 60°C for 30 seconds (exons 4 and 5) or 55°C (exon 3) and 72°C for 45 seconds followed by a final extension at 72°C for 5 minutes. SSCP analysis was performed as described in Chapter 2.

7.2.3 Sequencing

Sequencing of PCR fragments with abnormal SSCP patterns was performed as described in Chapter 2.

7.2.4 Cloning

Cloning of PCR fragments with suspected mutations was performed as described in Chapter 2.

7.2.5 Mutation specific enzyme digests

Mutations were confirmed using restriction enzymes with a cutting site which was created or removed in mutant alleles (Table 7.3). Digested PCR products were electrophoresed through 2.5 or 3.5% agarose gels and bands visualised by ethidium bromide staining. For each mutation 100 DNA samples from haematologically normal

Table 7.1 Sequences of PCR primers used for SSCP and restriction enzyme digest of *AML1* exons 3, 4 and 5 and PCR primers for LOH analysis. For primer 5MM the mismatch is underlined.

Primer	Sequence (5'→3')
3F*	ATCCCAAGCTAGGAAGACCGAC
3R*	TGTTTGCAGGGTCCTAACTCAATC
4F*	CATTGCTATTCCTCTGCAACC
4R*	CCATGAAACGTGTTTCAAGC
5F*	CCACCAACCTCATTCTGTTT
5R*	AGACATGGTCCCTGAGTATA
5MM	CACAGGGAAAAGCTTCACTCTGACGA
D21S65F**	CCGAAAACTTACTGGAGAAC
D21S65R**	GATCATCCAGGAATCACCAA
D21S270F**	GAAATGTTTTAATAAATGGTGGTTA
D21S270R**	ACAAAGTTATGGTCAAGGGG

* Primer sequences derived fron Song *et al* (1999)

** Primer sequences derived from Preudhomme *et al* (2000)

controls were also screened by specific enzyme digest or by size discrimination. For patient 7 it was necessary to use a mismatch primer 5MM (Table 7.1) which introduced a *Mbo* I cutting site in wild type alleles.

7.2.6 Loss of heterozygosity (LOH) analysis

LOH was analysed using primers for the highly polymorphic markers D21S65 and D21S270 which are centromeric and telomeric respectively to the *AML1* gene (Preudhomme *et al,* 2000; Table 7.1). PCR was performed as described above using a γ-^{32}P-end labelled primer but with an annealing temperature of 58°C and 30 cycles of amplification. Products were electrophoresed through denaturing polyacrylamide gels (7 M urea, 6% polyacrylamide, 0.5 X TBE, crosslinker ratio 37.5:1) which were subsequently dried and exposed to Hyperfilm-MP.

7.3 Results

7.3.1 Mutation detection

PCR-SSCP analysis of exons 3, 4 and 5 of the *AML1* gene and their flanking intron/exon boundaries was performed on DNA from 61 AML patients and the patterns obtained were compared with those using the haemopoietic cell line TF-1. Abnormalities were detected in 7 patients, one of whom had altered patterns in 2 different PCR fragments. Details of these patients are given in Table 7.2; 6 were FAB type M0 (6/41, 14%) and 1 was FAB type M7 (1/20, 5%). Patients 1-5 (Table 7.2) had abnormal patterns in the fragment encompassing exon 3 (Figure 7.2a-e), patients 5 and 6 in the exon 4 fragment (Figure 7.2f-g), and patient 7 in the exon 5 fragment (Figure 7.2h). Each particular abnormal pattern was observed in just one patient, and wild type bands were prominent in addition to the extra or shifted bands in all patients

Figure 7.2 Eight abnormal SSCP patterns were observed in 7 patients (P1-P7, A-H). Arrows indicate shifted or abnormal bands as compared with TF-1 (T). In patient 3 the abnormality was detected as double stranded heteroduplex (lower panel in C).

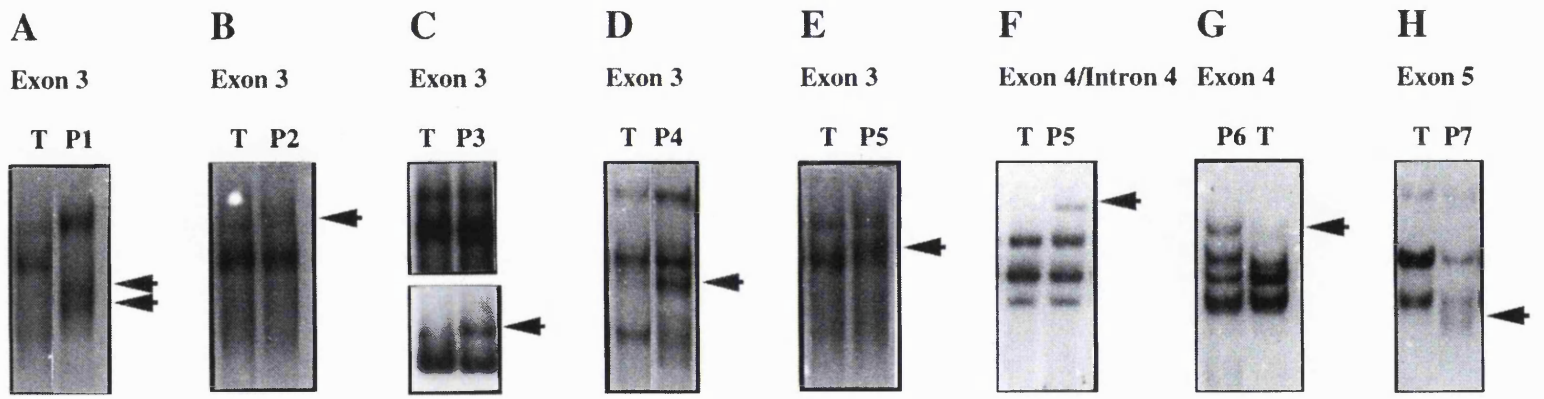


Table 7.2 Patient characteristics and details of *AML1* mutations.

Patient	Age(yrs)/ Sex	Sample type	FAB type	Cytogenetics	Location of mutation	Mutation*	Restriction enzyme confirmation	Predicted protein change
1	31/F	BM	M0	46,XX [10]	Exon 3	$G^{1815}\rightarrow T$	*Hin*P1 I	W79C
2	34/M	BM	M0	47,XY,+13 [23] / 46,XY [7]	Exon 3	$T^{1664}\rightarrow C$	*Bsa*H I	L29S
3	66/M	BM	M0	46,XY,der(4) [1] / 46,XY [9]	Exon 3	delC codon 70	*Mnl* I	Stop110
4	62/F	BM	M0	48,XX+2,+13 [4] / 46,XX [16]	Exon 3	insA codon 67	*Mwo* I	Stop110
5	62/M	BM	M0	47,XY,+13 [23] / 46,XY [7]	(i) Exon 3	$C^{1641}\rightarrow T$	*Hinf* I	Silent S21
					(ii) Exon4 / Intron 4	ins14bp	*Hph* I	**
6	55/M	PB	M7	N/A	Exon 4	ins18bp	Size	InsELRNAT115
7	19/M	PB	M0	45,XY,-7 [9] / 46,XY [1]	Exon 5	$T^{2027}\rightarrow C$	Mismatch primer + *Mbo* I	I150T

* corresponding to GenBank entry D43968

** introduction of alternative splice site (see text and Figure 7.2)

N/A not available

except patient 1, suggesting the presence of heterozygous mutations in the majority of patients. It is noteworthy that 3/6 patients with AML M0 and abnormal SSCP patterns had trisomy 13, a relatively common finding in this FAB type (Mehta *et al,* 1998).

Using direct sequencing of PCR products (see Chapter 2), 4 of the alterations were shown to be point mutations (Table 7.2). In patient 1, a $G^{1815}\rightarrow T$ substitution (numbered according to GenBank entry D43968) would lead to a W79C alteration, and patient 2 had a $T^{1664}\rightarrow C$ substitution which would cause a L29S alteration. In patient 5, the $C^{1641}\rightarrow T$ would not change the serine residue at position 21 (silent S21) and in patient 7, the $T^{2027}\rightarrow C$ would lead to I150T. For the other 4 alterations, PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Groningen, NL) and at least 3 clones with the same abnormal bands on SSCP analysis as the original patient sample were sequenced. Patient 3 had a single nucleotide deletion in codon 70 and patient 4 a single nucleotide insertion in codon 67. Both of these alterations would be predicted to cause a frameshift leading to introduction of a premature stop codon at amino acid 110. Patient 5 had an internal tandem duplication (ITD) of 14 nucleotides which included 12bp from the 3' end of exon 4 and the GT splice donor site of intron 4 (Figure 7.3). Although splicing at the normal position would still be possible (AGgtgaag...), the more favoured splice site would now be 14bp downstream of this (AGgtacgtt...) (Lewin, 2000) which would result in a frameshift after R142 and creation of a stop codon after 10 amino acids. Patient 6 had an 18bp ITD (insGACCTGAGAAATGCTACC) which would be predicted to insert 6 additional amino acids after A115. The insertion in patient 6 was clearly visible using agarose gel electrophoresis of the PCR product (Figure 7.4g). All other alterations were confirmed by restriction enzyme digestion of PCR products created using the relevant primer set (Table 7.3, Figure 7.4a-f) or a mismatch primer (patient 7, Figure 7.4h).

Figure 7.3 A: Introduction of an alternative splice site created by the ITD (italics) in patient 5. B: The ITD causes a frameshift resulting in termination of AML1 sequence in exon 5.

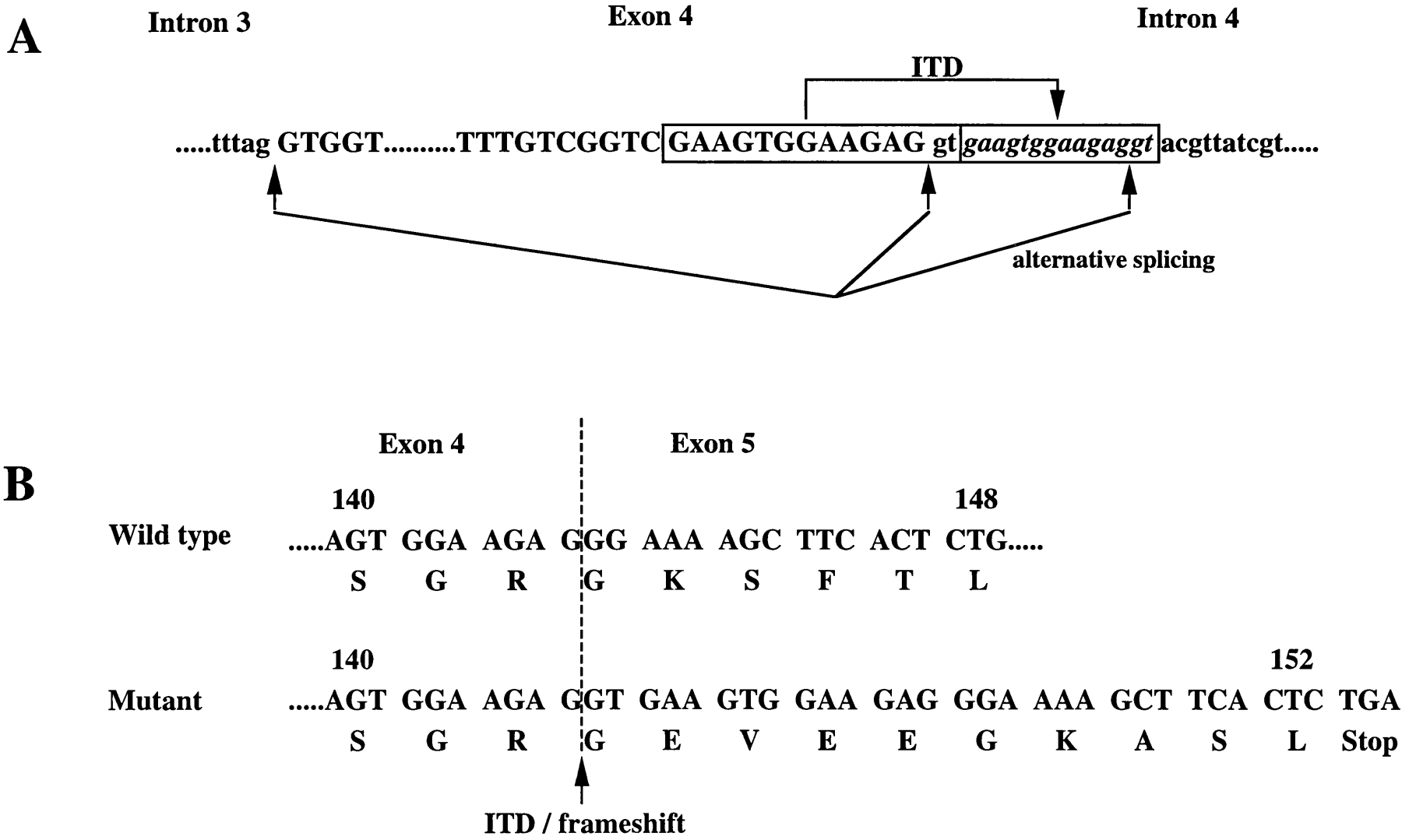


Table 7.3 Follow up and LOH analysis of patients with *AML1* mutations and HNCs.

Patient	Predicted protein change	Mutation at remission	Mutation at relapse	Frequency in 100 HNC	Wild type bands on SSCP	LOH for D21S65	LOH for D21S270
1	W79C	Absent	N/A	0%	Absent	Y	Y
2	L29S	N/A	N/A	5%	Present	NI	NI
3	Stop110	Absent	Present	0%	Present	N	N
4	Stop110	Absent	N/A	0%	Present	N	N
5	(i) SilentS21	Present	Present	3%	Present	NI	N
	(ii) ins14bp	Absent	Present	0%	Present	"	"
6	insELRNAT	N/A	N/A	0%	Present	NI	NI
7	I150T	N/A	N/A	0%	Present	-	-

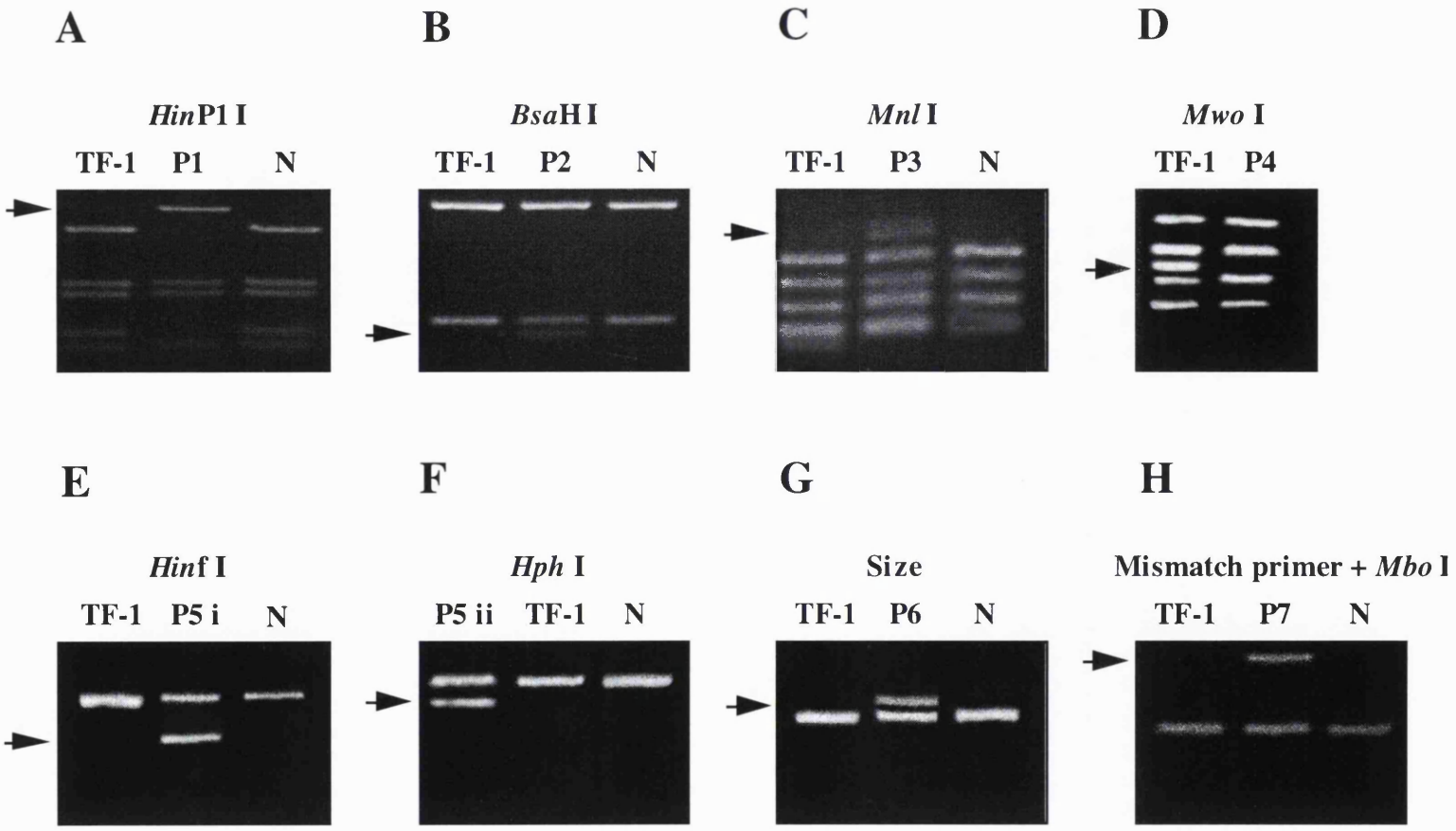
N/A sample not available

Y loss of heterozygosity

N no loss of heterozygosity

NI not informative.

Figure 7.4 Confirmation of the mutations detected using size or restriction enzyme digestion of PCR fragments. Arrows indicate the extra bands. P: patient; N: HNC.



7.3.2 Analysis of samples from patients in haematological remission and relapse and haematologically normal controls

DNA was available from bone marrow of 4 patients in haematological remission and 2 at relapse and was analysed using the mutation-specific restriction enzyme digests. Four of the five alterations, those in patients 1, 3, 4 and mutation (ii) in patient 5 were absent in the remission samples (Table 7.3). These results indicate that in these patients the mutations were acquired as part of their disease. In patients 3 and 5 the mutations reappeared at relapse. The silent S21 alteration observed at presentation in patient 5 was also present both in remission and relapse samples which was compatible with the view that it was a polymorphism.

Using either PCR fragment size discrimination or mutation-specific restriction enzyme digestion, 100 HNCs were screened for the presence of the mutations. The silent S21 and L29S substitutions were observed in 3% and 5% HNCs respectively. This data, together with the observation of the silent S21 in patient 5 in remission, indicated that these 2 substitutions were infrequent polymorphisms.

7.3.3 LOH analysis

LOH analysis was possible in 4 of the 6 patients deemed to have an acquired mutation who were informative for one or both of the highly polymorphic markers D21S65 and D21S270 and had remission DNA available (Figure 7.5). Patient 6 was not polymorphic at either locus and there was insufficient DNA to perform analysis in patient 7. LOH at presentation was detected only in patient 1, in whom no wild type *AML1* had been detected on SSCP analysis (Table 7.3).

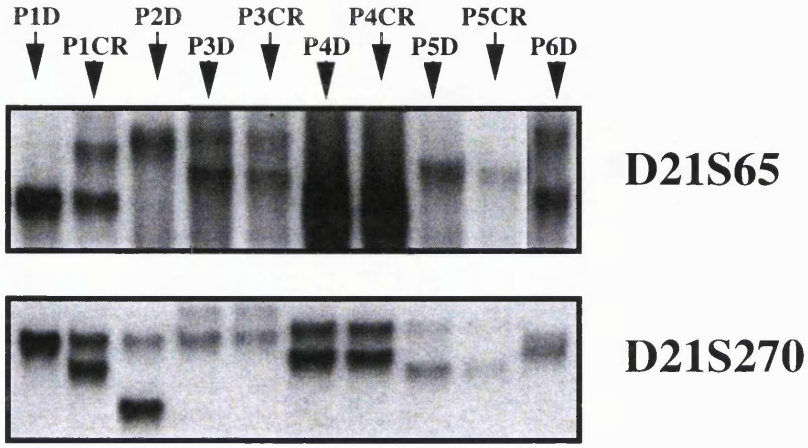


Figure 7.5 LOH analysis using the polymorphic, microsatellite markers D21S65 (centromeric) and D21S270 (telomeric). D: diagnosis; CR: complete remission.

7.4 Discussion

The transcription factor *AML1* is known to play an important role in the regulation of normal haemopoiesis, and there is increasing evidence that disruption of this gene is widely implicated in both myeloid and lymphoid leukaemogenesis (Downing, 2001). In addition to t(8;21), *AML1* has also been shown to be involved in other translocations in leukaemia: t(12;21) which occurs in approximately 25% of paediatric B cell ALL (Rubnitz *et al,* 1999), t(3;21) seen in occasional cases of secondary AML and blast crisis CML (Zent *et al,* 1996), and in at least 10 other rare translocations often observed in secondary AML (Berger *et al,* 1996; Roulston *et al,* 1998; Hromas *et al,* 2000; Richkind *et al,* 2000). Furthermore, several groups have now identified point mutations, insertions or deletions in the runt domain of *AML1* in patients with *de novo* and secondary AML (Osato *et al,* 1999; Preudhomme *et al,* 2000) and also MDS (Imai *et al,* 2000) which may contribute to leukaemogenesis. Of particular note was the presence of such mutations in 24% (12/50) of patients with the minimally differentiated FAB type M0. Functional studies have indicated that some of these mutations have deleterious effects on DNA and/or CBFβ binding which would result in dysregulation of genes under the control of AML1 (Osato *et al,* 1999; Imai *et al,* 2000).

In the present study, SSCP analysis identified abnormalities of *AML1* in 6 of the 41 patients with AML FAB type M0. One of these patients (patient 5) had 2 sequence changes. However, 2 of the substitutions, the silent S21 and L29S, are upstream of the runt domain and are non-pathological polymorphisms as indicated by their presence in 3% and 5% of control samples respectively, and by the demonstration of the silent S21 in a remission sample. The silent S21 occurred in a patient with an additional mutation so overall 5 M0 patients (12%) had mutations predicted to result in AML1 proteins with impaired function. In patient 1 the introduction of a cysteine in the

W79C mutation may result in new bonds within the protein which might alter or stabilise a particular conformation. This area appears to be functionally important as loss of cysteine 81 results in a marked decrease in DNA binding, although it has no effect on CBFβ binding (Kurokawa *et al,* 1996; Akamatsu *et al,* 1997). Both the delC and the insA mutations in patients 3 and 4 would result in a truncated protein that is unlikely to bind DNA as it lacks 5 of the β strands involved in the S-type immunoglobulin conformation which AML1 takes up when it is bound to DNA (Berardi *et al,* 1999; Nagata *et al,* 1999). The 14bp duplication in patient 5 would be predicted to alter the splice site at the end of exon 4 and cause a frameshift that would result in loss of the transactivation domain (Figure 7.3). The I150T mutation in patient 7 is in a region of the runt domain that is important for heterodimerisation with CBFβ (Warren *et al,* 2000), although it is not known whether presence of threonine would interfere with this interaction. Structural analyses suggest that none of the previously reported heterozygous mutations of *AML1* in AML patients would be predicted to interfere with CBFβ binding (Nagata & Werner, 2001; Tahirov *et al,* 2001).

The frequency of mutations detected in patients with M0 AML (12%) was therefore lower than in the previous reports with 33% and 24% respectively (Osato *et al,* 1999; Preudhomme *et al,* 2000) but the difference is not significant. Pooling the results from this study with the previous 2 published reports gives an incidence of *AML1* mutations of 20% in M0 AML. It is noteworthy that Yeoh *et al* (2000) have reported an analysis in abstract form in which the incidence of mutations was 13%, which is very similar to the findings described here.

An important aim of this study was to determine whether *AML1* mutations in M0 AML were always either biallelic or monoallelic with loss of the wild type allele. In previous reports (Osato *et al,* 1999; Preudhomme *et al,* 2000) only 1/12 cases of mutations in this type of AML was apparently heterozygous, whereas in the present

study, the wild type allele was present at comparable levels to the mutant allele in 4/5 cases. A possible confounding issue is that the wild type allele could be contributed by contaminating non-leukaemic cells but in 2 cases (patients 3 and 5) the percentage of blasts exceeded 85% (88% and 90% respectively), excluding the possibility of significant contamination by non-leukaemic cells. In 1 case (patient 4) the blast count was 60% and in another (patient 7) this was not recorded so caution in the interpretation of these cases is necessary in the present study. Similarly, in the abstract by Yeoh *et al* (2000) only 1/5 of M0 patients with mutations in the runt domain of *AML1* had biallelic disease. In the present study, only patient 1 showed complete lack of wild type *AML1* and LOH.

Identification of nonsense mutations or intragenic deletion of 1 *AML1* allele in patients with FPD/AML has led to the hypothesis that haploinsufficiency of the transcription factor causes thrombocytopenia with predisposition to the acquisition of additional mutations that cause leukaemia (Song *et al,* 1999; Barton & Nucifora, 2000). The first search for *AML1* mutations in a sizeable cohort of patients with the rare M7 type of AML was therefore performed. Only 1/20 (5%) patients had a heterozygous mutation, similar to the frequency found in other non-M0 AML types. This mutation, an ITD of 18bp (patient 6) is predicted to alter AML1 function. The insertion of 6 amino acids (ELRNAT) immediately downstream of A115 is likely to be on the surface of the protein (Tang *et al,* 2000) and to either interfere with DNA binding and/or form new interactions through the introduction of highly charged side chains.

Analysis of all reported mutations occurring in the runt domain of *AML1* (Osato *et al,* 1999, Song *et al,* 1999; Imai *et al,* 2000; Preudhomme *et al,* 2000; Yeoh *et al,* 2000; present study) reveals that they are distributed throughout exons 3, 4 and 5. Separation of those mutations that result in amino acid substitutions and those that are predicted

to result in a truncated protein reveal that these types of mutations are clustered into distinct regions of the gene. Mutations predicted to result in a truncated protein would all lack the transactivation domain (Figure 7.6) and are also located on the CBFB heterodimerisation surface of the AML1 molecule (Nagata *et al*, 2001). Several of these mutations have been shown to have minimal DNA binding capacity as compared wth wild type AML1 (Osato *et al*, 1999; Imai *et al*, 2000) and therefore reduced or absent transactivational capacity. As to whether these mutant proteins are produced in these patients and if so, their stabilities within the blast cells remains to be determined. The substitution mutations (Figure 7.7) affect amino acid residues that are mainly located within the β sheets of AML1. These mutations often result in alteration of the highly charged arginine (R) or lysine (K) residues necessary for molecular integrity and conformational change upon CBFB binding (Tahirov *et al*, 2001). Analysis of these substitution mutants has shown diminished DNA binding (Osato *et al*, 1999; Imai *et al*, 2000).

At present, the number of patients studied with *AML1* mutations is too small to determine whether they also have the relatively good prognosis that is characteristic of AML patients with t(8;21) (Grimwade *et al,* 1998; Byrd *et al,* 1999) as loss of the transactivation domain from 1 allele may influence patient survival. Conversely, in the FPD/AML families the *AML1* mutations are thought to be loss-of-function mutations that predispose to leukaemia through haploinsufficiency for tumour suppression (Song *et al,* 1999). It should be noted that a significant proportion of the mutations described in patients with M0 AML are predicted to result in truncated proteins that are similar to those in FPD/AML. Three out of 6 mutations in the present study fell into this category, which is consistent with a total of 12/22 mutations in the other 3 series (Osato *et al,* 1999; Preudhomme *et al,* 2000; Yeoh *et al,* 2000). The FAB types and clinical outcome of FPD/AML patients have not been reported but the association of acquired *AML1* mutations with minimally differentiated leukaemia is of

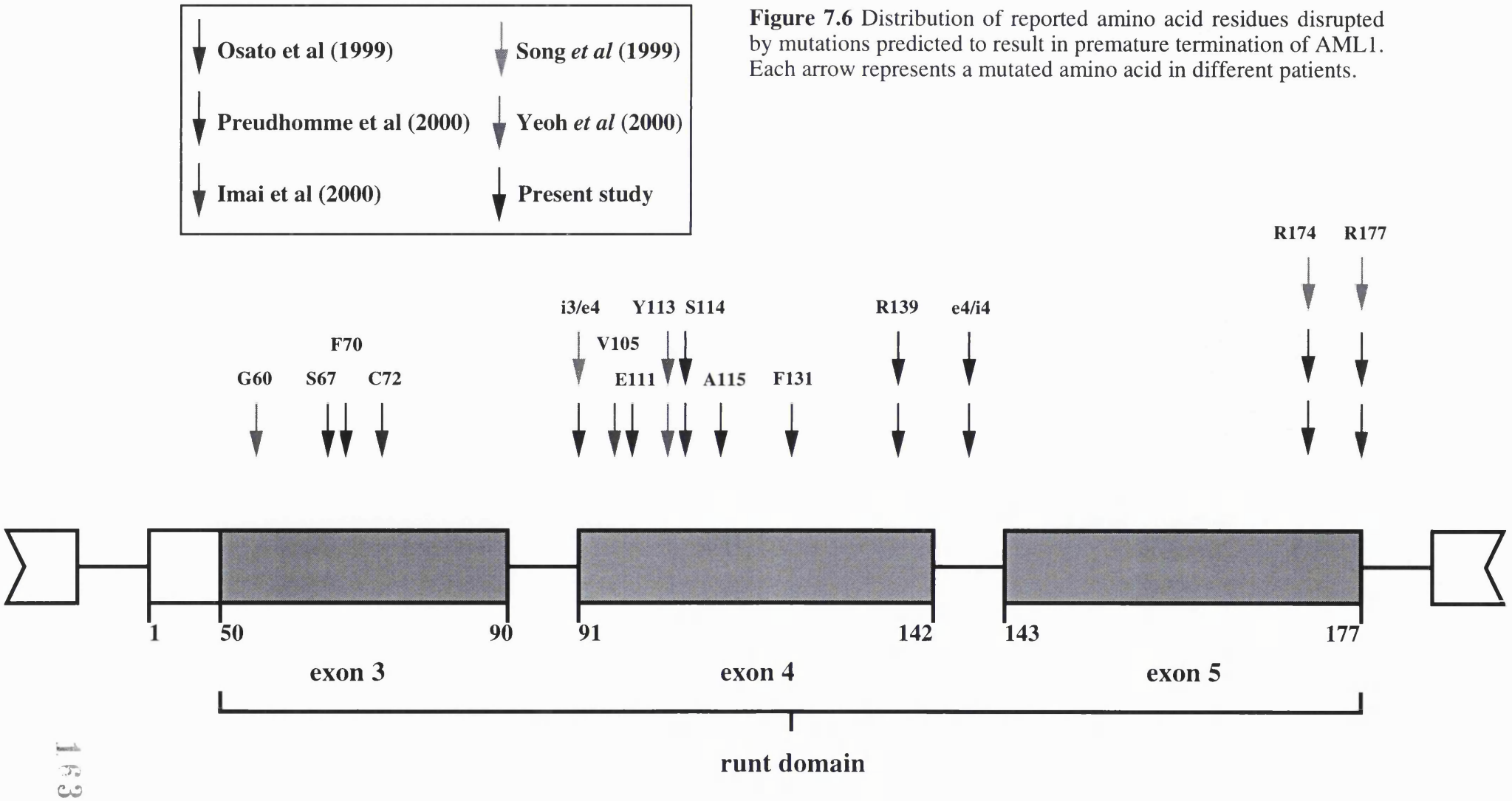


Figure 7.6 Distribution of reported amino acid residues disrupted by mutations predicted to result in premature termination of AML1. Each arrow represents a mutated amino acid in different patients.

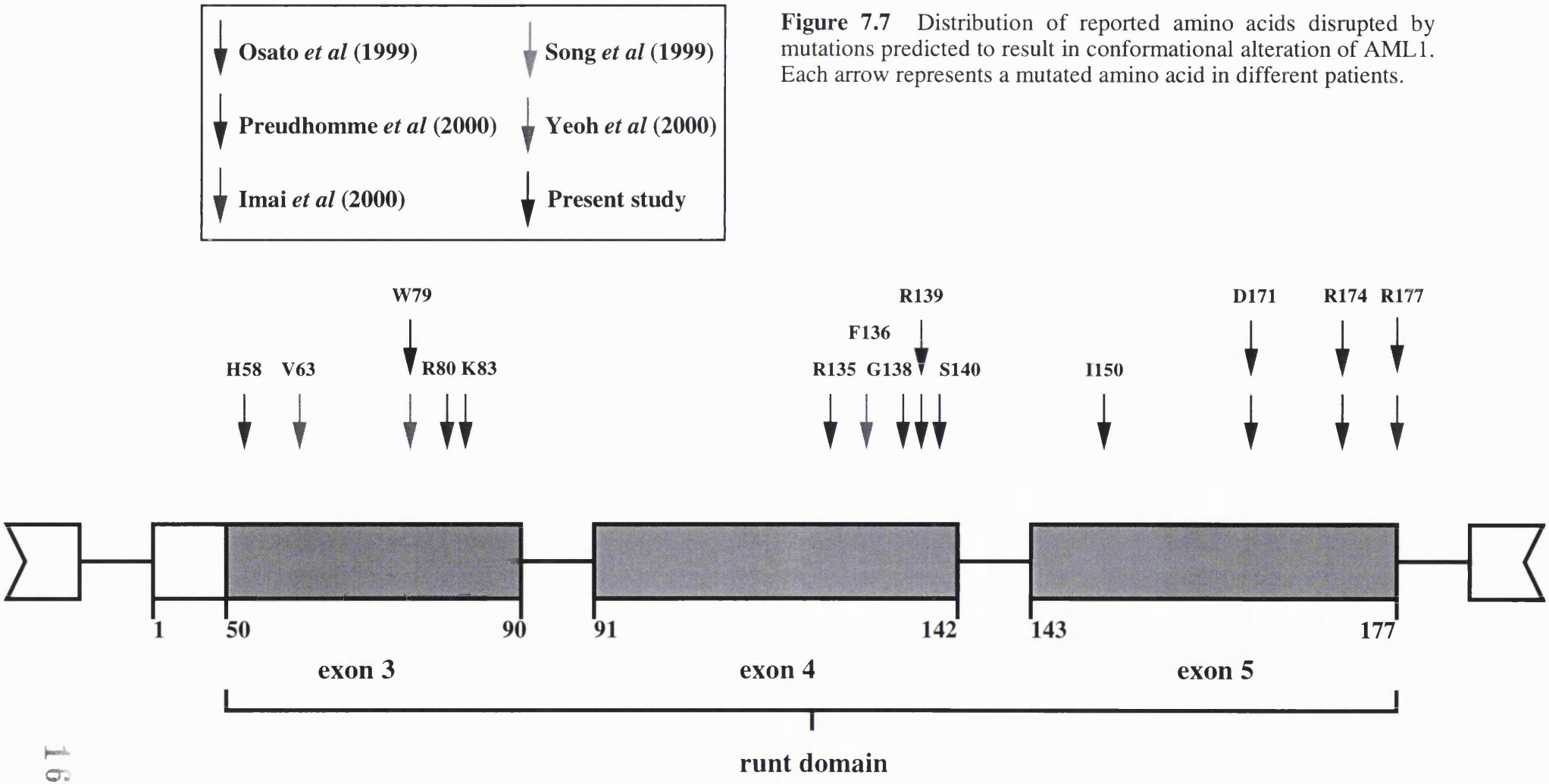


Figure 7.7 Distribution of reported amino acids disrupted by mutations predicted to result in conformational alteration of AML1. Each arrow represents a mutated amino acid in different patients.

interest. Since patients with FAB type M0 constitute <5% of all AML patients (Lowenberg *et al,* 1999), collective analysis of patients from several groups may be necessary to determine the impact of *AML1* mutations on long term outcome.

Chapter 8

Mutational analysis of *AML1* in FPD/AML

8.1 Introduction

Evidence for the multistep pathogenesis of human malignancies has come from families with an inherited predisposition to develop cancer. In some families, one mutation is inherited in the germline and subsequent mutations acquired in the other allele of the same gene give rise to the malignant phenotype, examples include Wilms tumour, retinoblastoma and familial colon cancer. Linkage analysis of certain pedigrees has led to the identification of several genes that, when mutated, dramatically increase the likelihood of developing cancer during an individual's lifetime. In colon cancer these genes include *MSH2* and *APC* in families with hereditary nonpolyposis cancer and adenomatous polyposis coli respectively (Kinzler & Vogelstein, 1996).

However, linkage studies have been less fruitful in the evaluation of haematological malignancies because of the rarity of familial syndromes. Familial leukaemia can occur in the context of a syndrome in which the leukaemia is one component of the overall disease, for example the trisomy 8 syndrome, Fanconi's anaemia, Wiskott-Aldrich syndrome or Kostmann's syndrome. Familial leukaemia may also occur as an isolated 'pure' leukaemia which can be classified on the basis of its inheritance pattern and by the involvement of haemopoietic lineages (Horwitz, 1997), examples of which include childhood myelodysplasia with monosomy 7 and chronic lymphocytic leukaemia.

To date, most mutant genes associated with acute leukaemias have been identified by their involvement in acquired chromosomal translocations. In many cases, the consequence of these translocations in AML has been the expression of fusion transcripts which involve one or more DNA-binding proteins. Examples include the *PML* and *RARα* genes disrupted by the t(15;17) in acute promyelocytic leukaemia,

CBFB which is disrupted by the inv(16) associated with acute myelomonocytic leukaemia (see Chapter 3), fusion of *MLL* to several partner genes in 11q23 translocations found in monocytic leukaemias and *AML1* and *ETO* genes in the t(8;21) translocation (see Chapter 4). Recurrence of these translocations suggests they play a central role in the pathogenesis of AML.

Although these alterations appear to be necessary for the eventual development of AML, several observations suggest more than one mutation is required to give rise to the clinical phenotype of AML. Firstly, expression of fusion transcripts such as *AML1-ETO* has been demonstrated in patients in long term remission, even following alloBMT (Jurlander *et al*, 1996). Secondly, creation of transgenic mouse models of AML with fusion genes has shown that these knocked-in sequences result in chronic myeloproliferative disorders (He *et al*, 1997; Castilla *et al*, 1999; Rhoades *et al*, 2000). These observations suggest that fusion genes are required for leukaemogenesis but are not sufficient for an acute leukaemic phenotype. This is analogous to the acquisition of further mutations in the development of the accelerated phase and blast crisis of patients with chronic phase CML (Santucci *et al*, 1996). Thirdly, myelodysplastic syndromes are preleukaemic syndromes in which multistep progression to AML is documented by serial acquisition of cytogenetic abnormalities associated with progression of the disease (Willman, 1998). Finally, there are rare but informative families with an inherited predisposition to develop AML, analogous to other forms of heritable malignancies (Horwitz *et al*, 1996a).

A specific disease entity was first noted by Luddy *et al* (1978) who described 3 siblings with a lifelong history of a bleeding disorder and thrombocytopenia who all died from a myeloid malignancy. The mother and other siblings had a variety of haematological abnormalities including chronic thrombocytopenia and abnormal platelet function. A further 2 pedigrees with similar abnormalities have subsequently

been described. At least 22 members of one large family were noted to have a bleeding tendency resulting from an autosomal dominant disorder of platelet production and function (Dowton *et al*, 1985). Phenotypic manifestations included mild to moderate thrombocytopenia, prolongation of the bleeding time and abnormal platelet aggregation. Six family members reportedly developed haematological malignancies, 4 of which were possibly AML. A family with an inherited bleeding disorder extending over 4 generations with multiple cases of myeloblastic and myelomonocytic leukaemia were studied by Gerrard *et al* (1991). In this family, bleeding abnormalities preceded 4 cases of AML by several years. The bleeding disorder was characterised by a prolonged bleeding time, abnormal platelet aggregation, low platelet ADP and decreased numbers of platelet dense bodies.

Ho *et al* (1996) reported linkage analysis of the pedigree previously described by Dowton *et al* (1985). Linkage was demonstrated to markers on chromosome 21q22 and 2 flanking markers were shown to define a critical region of the disease locus. Genes of interest in this region that were identified as potentially causal for this familial platelet disorder with a propensity to develop AML (FPD/AML) were *AML1*, *IFNAR1* encoding the interferon αβ receptor and *CRFB4* which encodes a typical member of the class II cytokine receptor family. Subsequent northern and Southern blotting, RNase protection, SSCP, direct sequencing and gel-shift analyses excluded the possibility of *AML1* being the gene responsible for FPD/AML (Legare *et al*, 1997). The observation that the FPD/AML locus was mapped to chromosome 21q22.1-2 was further extended by a report describing a new family with a similar phenotype (Arepally *et al*, 1998). Linkage analysis identified a locus that overlapped the same chromosomal region as that described by Ho *et al* (1996). This suggested that a defect in a single gene may underlie the FPD/AML phenotype.

Further mutational analysis of candidate genes identified nonsense mutations or intragenic deletion of one allele of *AML1* that co-segregated with the disease in 4 FPD/AML pedigrees (Song *et al*, 1999). Heterozygous missense mutations of the *AML1* gene that co-segregated with the disease were identified in a further 2 pedigrees. Analysis of bone marrow or peripheral blood cells from affected FPD/AML individuals showed a decrease in megakaryocyte colony formation, demonstrating that *AML1* dosage affects megakaryopoiesis. The findings support a model for FPD/AML in which haploinsufficiency of *AML1* causes an autosomal dominant congenital platelet disorder which predisposes to the acquisition of additional mutations that lead to leukaemia.

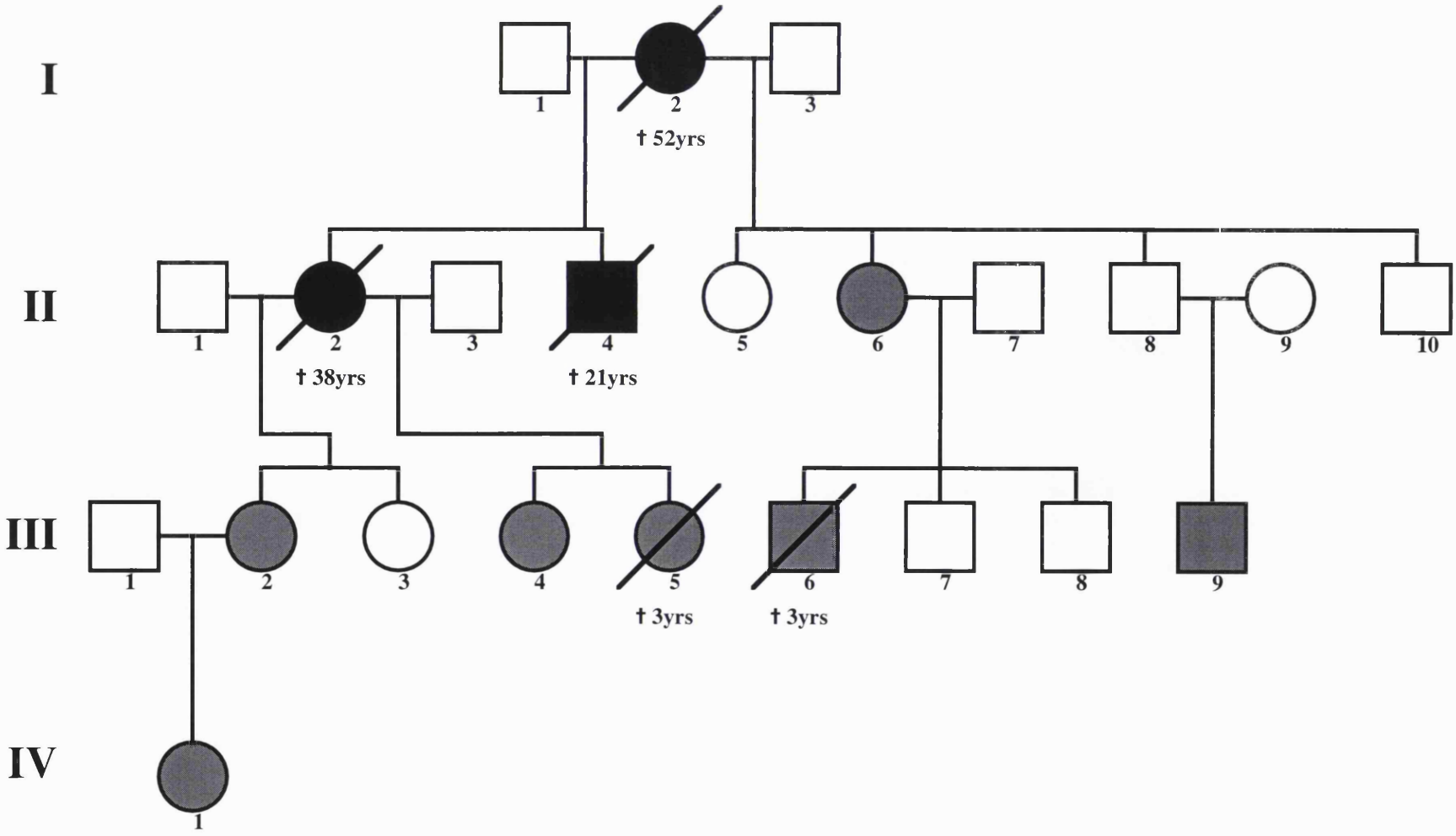
In this chapter, results are presented on a family with an FPD/AML phenotype in whom screening of 2 affected members identified a mutation of the *AML1* gene. In order to determine a complete family history, further clinical details of affected individuals were sought. At this time, it came to attention that this family had been previously characterised and reported as pedigree 5 by Song *et al* (1999). The mutation was given as R201Q, a difference of 27 amino acid residues, because of numbering from alternative start codons.

8.2 Materials and methods

8.2.1 Patients

The index case (II-2, Figure 8.1) presented at the age of 27 years after a miscarriage and was found to have thrombocytopenia (95 x 10^9/L, normal range 150 - 450 x 10^9/L) and abnormal platelet aggregation. Ten years later she developed MDS which subsequently transformed into AML (cytogenetics 47, XX,+8 in all cells) and she died from respiratory complications at age 38 years. The following family history was

Figure 8.1 Pedigree of FPD/AML family. ● : individuals who developed AML and ◍ : affected individuals with either MDS or thrombocytopenia.



compiled from the medical notes of the index case and other family members. Her mother (I-2) had previously died of AML at the age of 58 years. She had 1 sibling who died of AML at the age of 21 years (II-4), and 4 half-siblings, one of whom has MDS with thrombocytopenia (II-6) and whose son died after BMT for aplastic anaemia at 3 years of age (III-6). The son of another of the her half siblings has developed thrombocytopenia (III-9). Three of the 4 daughters of the index cases' are or were affected: one has thrombocytopenia (III-4), one died at age 3 years following a MUD transplant for refractory anaemia with ringed sideroblasts (RARS) (III-5) and another has MDS (III-2). This patient (III-2) has subsequently had a child who has developed thrombocytopenia (IV-1).

8.2.2 Samples

DNA was archived from the index case (II-2) at the time of transformation of MDS into AML and had been extracted from bone marrow mononuclear cells, peripheral blood, skin and cells obtained from a buccal scrape. DNA was extracted from an archival, iron stained, bone marrow smear from patient III-5 at the time of diagnosis of RARS using the method of Fey *et al* (1997) (see Chapter 2).

8.2.3 PCR analysis and sequencing

DNA from the bone marrow of both patients was used for PCR analysis. Fragments encompassing exons 3, 4 and 5 of the runt domain encoding region of the *AML1* gene were amplified using intronic PCR primers as described in Chapter 7.2.3. PCR products were cleaned and directly sequenced (see Chapter 2).

8.3 Results

A G^{2108}→A exon 5 substitution (numbered according to GenBank entry D43968) of the *AML1* gene was detected in the BM from patients II-2 and III-5. This is predicted to lead to an R174Q alteration at the protein level. The restriction enzyme *Ava* I cuts the wild type but not the mutant sequence. Digestion of PCR products confirmed the presence of the heterozygous mutation in the bone marrow cells of the 2 patients and also demonstrated the mutation in cells from the skin, peripheral blood and the buccal scrape of patient II-2 (Figure 8.2). DNA from 100 haematologically normal controls was screened by PCR and *Ava* I restriction enzyme digest for the presence of the substitution. No mutation was found in these normal controls.

8.4 Discussion

A family is described with FPD/AML and a mutation of the *AML1* gene. Presence of the R174Q mutation in the skin and buccal mucosa indicates that it is of germline origin. This R174Q mutation has also been described in a patient with AML M0 and trisomy 21 secondary to essential thrombocythaemia (Preudhomme *et al*, 2000). Functionally it would be predicted to result in loss of the DNA binding ability of one allele of AML1. Mutagenesis and structural studies have shown that this arginine residue is located in a region of the AML1 structure that is critical for DNA binding (Kagoshima *et al*, 1996; Berardi *et al*, 1999). Using an electrophoretic mobility shift assay, Osato *et al* (1999) demonstrated an R177Q mutation almost completely abolished DNA binding. Although further biochemical analysis will be required to characterise the DNA binding activity of the R174Q mutation, it is unlikely that this mutation would result in an AML1 protein with a dominant negative effect.

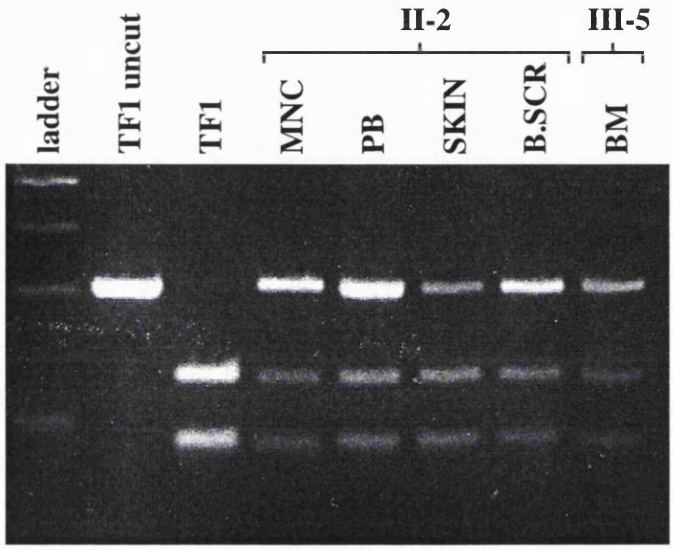


Figure 8.2 *Ava*I digest of exon 5 PCR products to detect R174Q mutation in FPD/AML affected members. BM MNC: bone marrow mononuclear cells; PB: peripheral blood; B.SCR: buccal scrape.

Of interest to note is the 'anticipation' of the leukaemia in this pedigree. Anticipation is the observation of increasing severity, or, as is the case in this family, earlier age of onset occurring with each passing generation in an inherited disease (Horwitz *et al*, 1996b). This phenomenon has been previously described in a number of non-haematological inherited disorders. Anticipation has been attributed to the expansion and generational accumulation of trinucleotide repeat sequences found in disease related genes (Sutherland & Richards, 1995). This mechanism has been implicated in the anticipatory effects observed in many inherited disorders including CCG expansion in the *FMR1* gene involved in Fragile X syndrome (Kremer *et al*, 1991; Verkerk *et al*, 1991), AGC repeats in myotonic dystrophy and neurodegenerative disorders such as Huntington's disease (La Spada *et al*, 1991; Huntington's Disease Collaborative Research Group, 1993; Nagafuchi *et al*, 1994), and also in familial chronic lymphocytic leukaemia (Auer *et al*, 2001; Wiernik *et al*, 2001). Several candidate leukaemia genes contain repetitive sequence motifs, particularly trinucleotide repeats, including *AF9*, a partner of MLL in the t(9;11)(p22;q23), *BCR* on chromosome 22q11 and a fragile site located at 16q22. As no trinucleotide repeat sequences have been found in the *AML1* gene (Hattori *et al*, 2000; Levanon *et al*, 2001) it is unlikely that this mechanism is the cause of anticipation in this FPD/AML pedigree. It is therefore likely that other mechanisms are involved in anticipation in familial malignancy syndromes.

Anticipation has also been observed in families with hereditary breast cancer and nonpolyposis colorectal carcinoma in which the genes responsible do not contain any repetitive sequence elements. A clue to the possible mechanism of anticipation comes from the knowledge that these genes function in DNA repair and genomic fidelity. Mutations of these genes would therefore precipitate the acquisition of downstrean mutations resulting in tumour development. It could possibly be hypothesised that these secondary mutations which result from such genomic instability may not be

acquired but could occur in the germline and could be transmitted to children. Leukaemia would occur after breaching a "threshold" of accumulated inherited and secondary mutations, with the progressive accumulation of mutations across generations accounting for the anticipation. The disruption of *AML1* in FPD/AML may possibly represent one of these inherited secondary mutations and implies the existence of a yet undefined mutated gene responsible for genetic stability in this disorder. The variable spectrum of the secondary mutations acquired after disruption of *AML1* might account for the clinical variability of MDS/AML subtypes in family members.

Inheritance of one mutant copy of a tumour suppressor gene (TSG) may predispose an individual to cancer as loss of the remaining wild type allele results in cells completely devoid of that gene product (Fearon, 1997). However, it is possible that haploinsufficiency of TSGs, i.e. expression at half normal levels due to only one functional allele, cannot suppress tumour growth. Haploinsufficient tumour suppression by $p27^{kip1}$ has recently been demonstrated in a mouse model (Fero *et al*, 1998) and in human ALL (Komuro *et al*, 1999). For those FPD/AML families so far described (Song *et al*, 1999) either one *AML1* allele is deleted or mutated, probably resulting in a loss of function. As one allele is retained it is possible that *AML1* is haploinsufficient for tumour suppression, suggesting predisposition to leukaemia without biallelic activation (Largaespada, 2001). A spectrum of cytogenetic abnormalities has been associated with progression to leukaemia in different FPD/AML individuals, including deletions of 5q, 7q and 20q, monosomy 7 and 11q23 translocations (Dowton *et al*, 1985; Ho *et al*, 1996; Arepally *et al*, 1998; Song *et al*, 1999) and also the trisomy 8 of the index case (II-2) described here, all of which are common chromosomal abnormalities in *de novo* AML. It is therefore plausible that haploinsufficiency of *AML1* predisposes to acquisition of secondary mutations at the aforementioned loci, although sequence analysis of *AML1* in several cases of MDS

and AML with partial or complete monosomy 5 or 7 has not revealed any mutations (Ferrari *et al*, 2001). It has therefore become more important to consider the possibility of allele loss as a cause of this effect in addition to mutations within the candidate haploinsufficient TSG.

If some TSGs are insufficient to fully suppress tumour growth in the remaining wild type copy, it may be possible to increase its expression or the activity of its protein product to achieve a therapeutic effect. Further studies in FPD/AML individuals who progress to AML will be necessary to address the role of haploinsufficiency of *AML1*.

Chapter 9

Conclusions

9.1 Conclusions

Treatment of AML consists of a standard induction phase followed by various post-remission options whose intensities are based mainly on the patients' age and eligibility for BMT. While alloBMT is regarded as the best option for patients with an HLA-identical donor, controversies exist surrounding the choice between autoBMT and chemotherapy as consolidation. Before the 1990's, no biological features of the leukaemic cells influenced therapeutic decisions. However, during the last fifteen years, cytogenetic features have increasingly become regarded as indicators of response to therapy and clinical outcome in AML, and the karyotype at diagnosis has become a major factor when considering post-remission therapeutic decisions (Mandelli *et al*, 1998). Much of the knowledge of the genetic features of AML is derived from conventional karyotyping which has become a useful tool in the identification of genes involved in the pathogenesis of AML. The use of RT-PCR as a complementary technique for the detection of fusion genes created by translocations and inversions offers several advantages, including the possibility of detecting cases with apparently normal, complex or failed karyotypes, the dissection of further heterogeneity by identification of distinct breakpoints, and the more sensitive assessment of response to treatment and monitoring of MRD.

Over the past decade, it has become increasingly evident that disruption of the CBF transcriptional factor complex by several mechanisms contributes to leukaemogenesis in a significant number of patients with AML (Figure 1.6), and work presented in this thesis explores several aspects of CBF AML. Given that studies concerning the detection, monitoring and analysis of the genes involved may provide insights into the biology of CBF AML, it is hoped that this information will be of benefit in determining appropriate treatment strategies for patients with these diseases.

The t(8;21) and inv(16) chromosomal abnormalities are two of the most common found in the leukaemic cells of patients with AML at diagnosis. Several large clinical trials have found that these patients have a high rate of achieving CR but, more importantly, they have a relatively low RR, hence an improved OS, when compared to patients with a normal karyotype or other structural and numerical chromosomal abnormalities (Grimwade *et al*, 1998, Bloomfield *et al*, 1998, Slovak *et al*, 2000). The t(8;21) and inv(16) result in the creation of the transcriptionally active fusion genes *AML1-ETO* and *CBFB-MYH11* respectively. The proteins encoded by these fusion genes are thought to contribute to leukaemogenesis by inhibiting the wild type CBF transcription factor complex which regulates a number of genes essential for normal haemopoiesis (Friedman, 1999).

Cytogenetic analysis is the technique of choice to identify the t(8;21) and inv(16), but with the introduction of complementary RT-PCR detection, it has become increasingly apparent that in several patients the fusion transcripts are present without karyotypic evidence of the corresponding abnormality. RT-PCR screening for *AML1-ETO* and *CBFB-MYH11* transcripts was therefore performed to assess the incidence of these events in a large number of patients with AML. *AML1-ETO* and *CBFB-MYH11* transcripts were more evident than the corresponding cytogenetic abnormalities in the populations of AML patients studied, particularly in FAB types other than M2 and M4Eo respectively. Of the AML patients studied, 6.5% had inv(16) whereas 10.3% had RT-PCR evidence of *CBFB-MYH11* fusion transcripts. *AML1-ETO* transcripts were detected in 12.9% of patients studied, although only 8.1% had cytogenetic evidence of t(8;21). A number of potential reasons for this discrepancy exist and they include cytogenetic failure due to insufficient or poor quality metaphases, reporting of false normal karyotypes due to analysis of residual normal populations, insertions in which the extent of the chromosomal material is below the resolution detected by conventional cytogenetic techniques or chromosomal

paints (Taviaux *et al*, 1999), complex rearrangements including 3-way translocations (Dierlamm *et al*, 1998), expression of transcripts by a minor leukaemic clone or even by normal marrow cell populations (Basecke *et al*, 1999) or by the reporting of false positives. The true incidence of these rearrangements may only be established by screening all new patients with AML regardless of FAB type. Whether the inv(16)-*/CBFB-MYH11+* and t(8;21)-*/AML1-ETO+* patients have the same relatively good prognosis as those patients with cytogenetic evidence of the corresponding abnormality remains unclear. These issues may only be addressed by RT-PCR evaluation of larger numbers of newly diagnosed cases of AML for *CBFB-MYH11* and *AML1-ETO* transcripts. This would allow a longer follow-up of similarly treated patients, enabling a more meaningful statistical analysis.

The identification of chromosomal translocations allows detection and quantitation of fusion transcripts providing an opportunity to use these transcripts as markers of MRD. RT-PCR techniques have been introduced that allow the detection of one leukaemic cell with the fusion gene, in up to 10^6 non-leukaemic cells, but as many leukaemia-associated fusion transcripts, including *AML1-ETO*, have been shown by these techniques to be present in patients in long-term remission, in some cases even after alloBMT, their value in predicting an impending relapse is limited. In order to address this problem, techniques have been introduced that allow the quantification of leukaemia-associated fusion transcripts. Among those currently employed are competitive RT-PCR and "real-time" RT-PCR which have been shown to be of value in quantifying a variety of transcripts (Hokland & Pallisgaard, 2000) but which have limited value in most laboratories due to practical reasons (construction of competitor templates, multiple testing of a single samples, reproducibility of sensitive techniques) or financial reasons (cost of an ABI Prism™ 7700 Sequence Detection System is approximately £70,000). A novel methodology, TMA/HPA, was therefore evaluated using the detection and quantification of *BCR-ABL* transcripts in CML as a

model system. This technique was shown to be linear, reproducible and had a sensitivity approximately equivalent to alternative methodologies, i.e. detection of one t(9;22) cell in less than 10^5 non-leukaemic cells. TMA/HPA could readily identify *BCR-ABL* positive samples and was shown to be of value in monitoring disease status after patients had undergone alloBMT, relapsed and had been treated with DLI. This strategy was subsequently applied to the detection of *AML1-ETO* transcripts in t(8;21) positive and negative patients with AML. The coupled TMA and HPA amplification and detection systems allowed the rapid identification of diagnostic samples bearing *AML1-ETO* transcripts in t(8;21)/t(8;V;21) and t(8;21)-/*AML1-ETO*+ patients as compared to HNC. This methodology also allowed the detection of one apparent false positive t(8;21)-/*AML1-ETO*+ sample, highlighting the importance of confirming the detection of leukaemia-associated fusion transcripts. Interestingly, in those patients with RT-PCR evidence of *AML1-ETO*, *AML1-ETO/ABL* ratios and *AML1-ETO* levels of the t(8;21)+ patients was approximately one log higher than that of the t(8;21)- patients. Whether this difference in *AML1-ETO* levels is a true biological phenomenon is unclear as the PB blast counts were unavailable and the median blast count of the t(8;21)+ BM samples was also higher than that of the t(8;21)- patients. Quantification of *AML1-ETO* levels in a greater number BM samples from t(8;21)-/*AML1-ETO*+ patients may resolve this apparent difference. The sensitivity of the *AML1-ETO* assay, i.e. detection of one t(8;21) cell in less than 10^4 non-leukaemic cells, was approximately one log less sensitive than that of the *BCR-ABL* assay. Nevertheless, retrospective analysis of t(8;21) AML patients at presentation and at follow-up permitted detection of an increase in *AML1-ETO* transcripts heralding relapse. TMA/HPA may therefore represent a viable alternative to existing quantitative methodologies as it is rapid, requires considerably smaller quantities of template RNA, does not require elaborate instrumentation, and is inherently designed to reduce contamination or crossover. Furthermore, it can be adapted to amplify and detect other translocation generated fusion genes.

Previous studies have described the presence of mutations of *AML1* in a proportion of patients with AML, particularly of the FAB type M0. A number of these mutations are heterozygous but in many patients, either mutations occur in the second allele or there is deletion of the other *AML1* allele (Osato *et al*, 1999; Preudhomme *et al*, 2000) suggesting lack of differentiation in a significant proportion of AML M0 patients may be due to a severe deficiency of functional AML1 protein. As *AML1* mutations have also been demonstrated in FPD/AML patients in whom there is impaired megakaryopoiesis, this study sought to confirm the high incidence of mutations in the runt domain of the *AML1* gene in M0 AML, to determine whether this FAB type is associated with biallelic or monoallelic disease, and to identify mutations in patients with the M7 subtype. Six mutations which would be predicted to result in impaired *AML1* function or a truncated protein were identified, together with two polymorphisms. The incidence in the M0 type (12%) was lower than the 33% (Osato *et al*, 1999) and 24% (Preudhomme *et al*, 200) previously reported. Mutations of *AML1* had not been previously investigated in AML patients with the FAB type M7, in which the incidence here was 5%. This is a similar frequency to that reported in FAB types other than M0. Only one of the patients with a mutation was shown by LOH analysis to have lost the wild type allele. This is contrary to previous findings (Osato *et al*, 1999; Preudhomme *et al*, 2000) in which 11/12 of AML M0 cases analysed were either biallelic or monoallelic with loss of the wild type allele. A feature of these reports is the small numbers studied, as patients with M0 constitute a minority (<5%) of all newly presenting cases of AML, which precludes reliable determination of the precise frequency of *AML1* mutations. Whether these patients have the same relatively good prognosis as t(8;21) AML patients, as loss of the transactivation domain from one allele may influence patient survival, is as yet unknown as the number of patients studied is too small for meaningful statistical analysis. Combining patient outcomes from several groups may provide an answer

but this approach may be hampered by the non-equivalent protocols used to treat the patients.

Of related interest was the demonstration of either mutations within, or loss of an *AML1* allele in the disorder of FPD/AML, previously characterised in only six pedigrees (Song *et al*, 1999). A family was identified with clinical features typical of FPD/AML present in four generations. Archival DNA from two family members was analysed. A germline mutation was identified in exon 5 of the *AML1* gene in both family members. Of note in this family, and not previously described in this disorder, was the anticipation of the leukaemia, i.e. the lower age of onset of AML in subsequent generations. In FPD/AML, *AML1* haploinsufficiency, i.e. expression at half normal levels due to only one functional allele, may predispose the patient to AML, as the remaining allele may not be able to suppress tumour growth. This is the first type of human malignancy described in which haploinsufficiency contributes to leukaemogenic transformation implying that allelic loss may occur more frequently in AML than previously thought.

9.2 Future directions

The molecular characterisation of genes involved in myeloid leukaemias has proved to be of particular value in APL and CML. Cloning of the *PML-RARα* gene, resulting from the APL associated t(15;17), has led to an unravelling of the mechanisms of ATRA containing treatment regimes that have vastly improved the CR and OS rates in patients with this type of AML (Fenaux *et al*, 1997). Similarly, an understanding of the disrupted tyrosine kinase activities of BCR-ABL in CML has led to the introduction of a novel therapeutic agent, STI571, that is able to reverse the actions of this oncoprotein (Druker *et al*, 2001b). This finding is particularly pertinent as receptor tyrosine kinases are a frequent target of mutation in several other types of

leukaemias and solid tumours (Boissan *et al*, 2000, Kottaridis *et al*, 2001; Zwick *et al*, 2001) and agents with similar inhibitory actions may be of value in the treatment of these malignancies (Morin, 2000). It is therefore hoped that further studies regarding the biology of CBF AML will be able to provide information enabling the development of specific therapeutic strategies, tailored to the molecular defect of the disease.

The ability of retinoic acid to overcome PML-RARα-mediated transcriptional repression, enabling the differentiation of APL promyelocytes, raises the hope that agents which prevent AML1-ETO from interfering with endogenous AML1 would also stimulate leukaemic differentiation or apoptosis and be of value in the therapy of patients with CBF AML. In t(8;21) AML, the ETO portion of the fusion protein has been shown to recruit a transcriptional repressor complex (see Chapter 1), thereby repressing transcription from genes normally activated by AML1. Theoretically, in the presence of HDAC inhibitors, chromatin structure will be relaxed and AML1-ETO repression relieved, allowing normal AML1 function to overcome the leukaemic differentiation block (Willman, 2001). Use of HDAC inhibitors such as phenylbutyrate and trichostatin A have, *in vivo*, been able to partially reverse ETO-mediated repression and induce a degree of differentiation in Kasumi-1 cells (Wang *et al*, 1999). Furthermore, HDAC inhibitors do not alone activate transcription. Such transcriptional activation also requires the presence of ATRA to induce the engagement of HATs, thereby acetylating adjacent histones, promoting chromatin to relax and allowing AML1 to bind and initiate transcription (Ferrara *et al*, 2001). Therefore, HDACs are likely to be used in conjunction with other activating compounds (e.g. ATRA) and chemotherapy. Other possible therapeutic strategies include either disruption of CBFB-MYH11-mediated cytoplasmic sequestration of AML1 (Kanno *et al*, 1998) or the interaction of AML1-ETO with corepressor complexes.

Advances in technology may provide the key to further understanding of CBF AML. Refinements in karyotyping technologies such as comparative genomic hybridisation and spectral karyotyping provide the promise of high resolution and automated screening for the chromosomal imbalances associated with CBF AML (Kim *et al*, 2001). Gene expression monitoring by DNA microarray analysis may also provide rapid identification of CBF AML subtypes and identify downstream targets of the associated rearrangements (Sasaki *et al*, 2000; Harada *et al*, 2001). Establishment of mouse models of CBF leukaemia will hopefully lead to the identification of mutations that co-operate with *AML1-ETO* and *CBFB-MYH11* in leukaemogenic transformation.

References

Adya N, Castilla LH & Liu PP (2000) Function of CBFβ/Bro proteins. *Seminars in Cell and Developmental Biology*, **11**, 361-368.

Ahn MY, Huang G, Bae SC, Wee HJ, Kim WY & Ito Y (1998) Negative regulation of granulocytic differentiation in the myeloid precursor cell line 32Dcl13 by ear-2, a mammalian homolog of Drosophila seven-up, and a chimeric leukemogenic gene, AML1-ETO. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 1812-1817.

Akamatsu Y, Ohno T, Hirota K, Kagoshima H, Yodoi J & Shigesada K (1997) Redox regulation of the DNA binding activity in transcription factor PEBP2: the roles of two conserved cysteine residues. *Jounal of Biological Chemistry*, **272**, 14497-14500.

Allan NC, Richards SM & Shepherd PC (1995) UK MRC randomised, multicentre trial of interferon alpha n1 for chronic myeloid leukaemia: improved survival irrespective of cytogenetic response. *Lancet*, **345**, 1392-1397.

Andrieu V, Radford-Weiss I, Troussard X, Chane C, Valensi F, Guesnu M, Haddad E, Viguier F, Dreyfus F, Varet B, Flandrin G & Macintyre E (1996) Molecular detection of t(8;21)/AML1-ETO in AML M1/M2: correlation with cytogenetics, morphology and immunophenotype. *British Journal of Haematology*, **92**, 855-865.

Arepally G, Rebbeck TR, Song W-J, Gilliland G, Maris JM & Poncz M (1998) Evidence for genetic homogeneity in a familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML). *Blood*, **92**, 2600-2602.

Arnold LJ Jr, Hammond PW, Wiese WA & Nelson NC (1989) Assay formats involving acridinium-ester-labeled DNA probes. *Clinical Chemistry*, **35**, 1588-1594.

Aronson BD, Fisher AL, Blechman K, Caudy M & Gergen JP (1997) Groucho-dependent and -independent repression activities of Runt domain proteins. *Molecular and Cellular Biology*, **17**, 5581-5587.

Arthur DC & Bloomfield CD (1983) Association of partial deletion of the long arm of chromosome 16 and bone marrow eosinophilia in acute non-lymphocytic leukemia. *Blood*, **62**, 931.

Asou H, Tashiro S, Hamamoto K, Otsuji A, Kita K & Kamada N (1991) Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8;21 chromosome translocation. *Blood*, **77**, 2031-2036.

Auer RL, Jones C, Mullenbach RA, Syndercombe-Court D, Milligan DW, Fegan CD & Cotter FE (2001) Role for CCG-trinucleotide repeats in the pathogenesis of chronic lymphocytic leukemia. *Blood*, **97**, 509-515.

Aventin A, La Starza R, Nomdedeu J, Brunet S, Sierra J & Mecucci C (2000) Typical CBFbeta-MYH11 fusion due to insertion of the 3' MYH11 gene into 16q22 in acute monocytic leukemia with normal chromosomes 16 and trisomies 8 and 22. *Cancer Genetics and Cytogenetics*, **123**, 137-139.

Avvisati G, Lo Coco F & Mandelli F (2001) Acute promyelocytic leukemia: clinical, morphological features and prognostic factors. *Seminars in Hematology*, **38**, 4-12.

Baer MR, Stewart CC, Lawrence D, Arthur DC, Byrd JC, Davey FR, Schiffer CA & Bloomfield CD (1997) Expression of the neural cell adhesion molecule CD56 is associated with short remission duration and survival in acute myeloid leukemia with t(8;21)(q22;q22). *Blood*, **90**, 1643-1648.

Bain BJ (1998) Morphology and classification of leukaemias. In: *Leukemia and related disorders*. Eds: Whittaker JA & Holmes JA, Blackwell Science, Oxford.
Barton K & Nucifora G (2000) AML1 haploinsufficiency, gene dosage, and the predisposition to acute leukemia. *Bioessays*, **22**, 214-218.

Basecke J, Jlussi M, Cepek L, Hildenhagen S, Brittinger G, Wormann B & Griesinger F (1999) AML1/ETO is transcribed in the cord blood and bone marrow of healthy individuals. *Blood*, **94**, 657a.

Behre G, Zhang P, Zhang D-E & Tenen DG (1999) Analysis of the modulation of transcriptional activity in myelopoiesis and leukemogenesis. *Methods*, **17**, 231-237.

Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR & Sultan C (1976) Proposals for the classification of the acute leukaemias. *British Journal of Haematology*, **33**, 451-458.

Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR & Sultan C (1985a) Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Annals of Internal Medicine*, **103**, 620-625.

Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR & Sultan C (1985b) Criteria for the diagnosis of acute leukemia of megakaryocyte lineage (M7). A report of the French-American-British Cooperative Group. *Annals of Internal Medicine*, **103**, 460-462.

Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR & Sultan C (1991) Proposal for the recognition of minimally differentiated acute myeloid leukaemia (AML-M0). *British Journal of Haematology*, **78**, 325-329.

Berardi MJ, Sun C, Zehr M, Abildgaard F, Peng J, Speck NA & Bushweller JH (1999) The Ig fold of the core binding factor alpha Runt domain is a member of a family of structurally and functionally related Ig-fold DNA-binding domains. *Structure, Folding and Design*, **7**, 1247-1256.

Berger R, Flandrin G, Bernheim A, Le Coniat M, Vecchione D, Pacot A, Derre J, Daniel MT, Valensi F & Sigaux F (1987) Cytogenetic studies on 519 consecutive de novo acute nonlymphocytic leukemias. *Cancer Genetics and Cytogenetics*, **29**, 9-21.

Berger R, Le Coniat M, Romana SP & Jonveaux P (1996) Secondary acute myeloblastic leukemia with t(16;21)(q24;q22) involving the AML1 gene. *Hematology and Cell Therapy*, **38**, 183-186.

Bitter MA, Le Beau MM, Larson RA, Rosner MC, Golomb HM, Rowley JD & Vardiman JW (1984) A morphological and cytochemical study of acute myelomonocytic leukemia with abnormal marrow eosinophils associated with inv(16)(p13q22). *American Journal of Clinical Pathology*, **81**, 733-741.

Bloomfield CD, Lawrence D, Byrd JC, Carroll A, Pettenati MJ, Tantravahi R, Patil SR, Davey FR, Berg DT, Schiffer CA, Arthur DC & Mayer RJ (1998) Frequency of prolonged remission duration after high-dose cytarabine intensification in acute myeloid leukemia varies by cytogenetic subtype. *Cancer Research*, **58**, 4173-4179.

Boissan M, Feger F, Guillosson J-J & Arock M (2000) c-kit mutations in mastocytosis and other hematological diseases. *Journal of Leukocyte Biology*, **67**, 135-148.

Borkhardt A, Harbott J & Lampert F (1999) Biology and clinical significance of the TEL/AML1 rearrangement. *Current Opinion in Pediatrics*, **11**, 33-38.

Bravo J, Li Z, Speck NA & Warren AJ (2001) The leukemia-associated AML1 (Runx1)-CBFβ complex functions as a DNA-induced molecular clamp. *Nature Structural Biology*, **8**, 371-378.

Bruhn L, Munnerlyn A & Grosschedl R (1997) ALY, a context-dependent coactivator of LEF-1 and AML-1 is required for TCRalpha enhancer function. *Genes and Development,* **11**, 640-653.

Buchner T, Hiddemann W, Schoch C, Haferlach T, Sauerland MC & Heinecke A (2001) Acute myeloid leukaemia (AML): treatment of the older patient. *Best Practice and Research Clinical Haematology*, **14**, 139-151.

Burnett AK, Goldstone AH, Stevens RM, Hann IM, Rees JK, Gray RG & Wheatley K (1998) Randomised comparison of addition of autologous bone marow transplantation to intensive chemotherapy for acute myeloid leukaemia in first remission: results of MRC AML 10 trial. *Lancet*, **351**, 700-708.

Burnett AK, Grimwade D, Solomon E, Wheatley K & Goldstone AH (1999) Presenting white blood cell count and kinetics of molecular remission predict prognosis in acute promyelocytic leukemia treated with all-trans retinoic acid: result of the randomized MRC trial. *Blood*, **93**, 4131-4143.

Burnett AK (2001) Treatment of acute myeloid leukaemia in younger patients. *Best Practice and Research Clinical Haematology*, **14**, 95-118.

Busson-Le Coniat M, Khac FN, Daniel MT, Bernard OA & Berger R (2001) Chromosome 21 abnormalities with AML1 amplification in acute lymphoblastic leukemia. *Genes Chromosomes and Cancer*, **32**, 244-249.

Byrd JC, Weiss RB, Arthur DC, Lawrence D, Baer MR, Davey F, Trikha ES, Carroll AJ, Tantravahi R, Qumsiyeh M, Patil SR, Moore JO, Mayer RJ, Schiffer CA & Bloomfield CD (1997) Extramedullary leukemia adversely affects hematologic complete remission rate and overall survival in patients with t(8;21)(q22;q22): results from Cancer and Leukemia Group B 8461. *Journal of Clinical Oncology*, **15**, 466-475.

Byrd JC, Dodge RK, Carroll A, Baer MR, Edwards C, Stamberg J, Qumsiyeh M, Moore JO, Mayer RJ, Davey F, Schiffer CA & Bloomfield CD (1999) Patients with t(8;21)(q22;q22) and acute myeloid leukemia have superior failure-free and overall survival when repetitive cycles of high-dose cytarabine are administered. *Journal of Clinical Oncology*, **17**, 3767-3775.

Calabrese G, Fantasia D, Franch PG, Morizio E, Stuppia L, Gatta V, Olioso P, Mingarelli R, Spadano A & Palka G (2000) Spectral karyotyping (SKY) refinement of a complex karyotype with t(20;21) in a Ph-positive CML patient submitted to peripheral blood stem cell transplantation. *Bone Marrow Transplantation*, **26**, 1125-1127.

Castilla LH, Wijmenga C, Wang Q, Stacy T, Speck NA, Eckhaus M, Marin-Padilla M, Collins FS, Wynshaw-Boris A & Liu PP (1996) Failure of embryonic hematopoiesis and lethal hemorrhages in mouse embryos heterozygous for a knocked-in leukemia gene CBFB-MYH11. *Cell*, **87**, 687-696.

Castilla LH, Garrett L, Adya N, Orlic D, Dutra A, Anderson S, Owens J, Eckhaus M, Bodine D & Liu PP (1999) The fusion gene Cbfb-MYH11 blocks myeloid differentiation and predisposes mice to acute myelomonocytic leukaemia. *Nature Genetics*, **23**, 144-146.

Chang KS, Fan YH, Stass SA, Estey EH, Wang G, Trujillo JM, Erickson P & Drabkin H (1993) Expression of AML1-ETO fusion transcripts and detection of minimal residual disease in t(8;21)-positive acute myeloid leukemia. *Oncogene*, **8**, 983-988.

Cheng T, Shen H, Giokas D, Gere J, Tenen DG & Scadden DT (1996) Temporal mapping of gene expression levels during the differentiation of individual primary hematopoietic cells. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 13158-13163.

Cherry AM, Bangs CD, Jones P, Hall S & Natkunam Y (2001) A unique AML1 (CBFA2) rearrangement, t(1;21)(p32;q22), observed in a patient with acute myelomonocytic leukemia. *Cancer Genetics and Cytogenetics*, **129**, 155-160.

Chomczynski P & Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, **162**, 156-159.

Claxton DF, Liu P, Hsu HB, Marlton P, Hester J, Collins F, Deisseroth AB, Rowley JD & Siciliano MJ (1994) Detection of fusion transcripts generated by the inversion 16 chromosome in acute myelogenous leukemia. *Blood*, **83**, 1750-1756.

Claxton D, Xie QS, Patel S, Deisseroth AB & Kornblau S (1996) The gene product of CBFB-MYH11. *Leukemia*, **10**, 1479-1485.

Claxton D & Choudhury A (2001) Potential for therapy with AML-derived dendritic cells. *Leukemia*, **15**, 668-669.

Cline MJ (1994) The molecular basis of leukemia. *New England Journal of Medicine*, **330**, 328-336.

Compton J (1991) Nucleic acid sequence-based amplification. *Nature*, **350**, 91-92.

Costello R, Sainty D, Blaise D, Gastaut JA, Gabert J, Poirel H, Buzyn-Veil A & Macintyre E (1997a) Prognosis value of residual disease monitoring by polymerase chain reaction in patients with CBFbeta/MYH11-positive acute myeloblastic leukemia . *Blood*, **89**, 2222-2223.

Costello R, Sainty D, Lecine P, Cusenier A, Mozziconacci MJ, Arnoulet C, Maraninchi D, Gastaut JA, Imbert J, Lafage-Pochitaloff M & Gabert J (1997b) Detection of CBFbeta/MYH11 fusion transcripts in acute myeloid leukemia: heterogeneity of cytological and molecular characteristics. *Leukemia*, **11**, 644-650.

Cross NC, Feng L, Chase A, Bungey J, Hughes TP & Goldman JM (1993) Competitive polymerase chain reaction to estimate the number of BCR-ABL transcripts in chronic myeloid leukemia patients after bone marrow transplantation. *Blood*, **82**, 1929-1936.

Crute BE, Lewis AF, Wu Z, Bushweller JH & Speck NA (1996) Biochemical and biophysical properties of the core-binding factor alpha2 (AML1) DNA-binding domain. *Journal of Biological Chemistry*, **271**, 26251-26260.

Daga A, Tighe JE & Calabi F (1992) Leukaemia/Drosophila homology. *Nature*, **356**, 484.

Dal Cin P, Atkins L, Ford C, Ariyanayagam S, Armstrong SA, George R, Cleary A & Morton CC (2001) Amplification of AML1 in childhood acute lymphoblastic leukemias. *Genes Chromosomes and Cancer*, **30**, 407-409.

Daley GQ, Van Etten RA & Baltimore D (1990) Induction of chronic myelogenous leukemia in mice by the $p210^{bcr\text{-}abl}$ gene of the Philadelphia chromosome. *Science*, **247**, 824-830.

Dann EJ & Rowe JM (2001) Biology and therapy of secondary leukemias. *Best Practice and Research Clinical Haematology*, **14**, 119-137.

Das-Gupta EP, Seedhouse CH & Russell NH (2000) DNA repair mechanisms and acute myeloblastic leukemia. *Hematological Oncology*, **18**, 99-110.

Dash A & Gilliland DG (2001) Molecular genetics of acute myeloid leukaemia. *Best Practice and Research Clinical Haematology*, **14**, 49-64.

Dastugue N, Payen C, Lafage-Pochitaloff M, Bernard P, Leroux D, Huguet-Rigal F, Stoppa AM, Marit G, Molina L, Michallet M, Maraninchi D, Attal M & Reiffers J (1995) Prognostic significance of karyotype in de novo adult acute myeloid leukemia-the BGMT group. *Leukemia*, **9**, 1491-1498.

Dauwerse JG, Wessels JW, Giles RH, Wiegant J, van der Reijden BA, Fugazza G, Jumelet EA, Smit E, Baas F, Raap AK (1993) Cloning the breakpoint cluster region of the inv(16) in acute nonlymphocytic leukemia M4 Eo. *Human Molecular Genetics*, **2**, 1527-1534.

Dauwerse HG, Smit EM, Giles RH, Slater R, Breuning MH, Hagemeijer A & van der Reijden BA (1999) Two-colour FISH detection of the inv(16) in interphase nuclei of patients with acute myeloid leukaemia. *British Journal of Haematology*, **106**, 111-114.

de Greef GE, Hagemeijer A, Morgan R, Wijsman J, Hoefsloot LH, Sandberg AA & Sacchi N (1995) Identical fusion transcript associated with different breakpoints in the AML1 gene in simple and variant t(8;21) acute myeloid leukemia. *Leukemia*, **9**, 282-287.

De Lord C, Powles R, Mehta J, Wilson K, Treleaven J, Meller S & Catovsky D (1998) Familial acute myeloid leukaemia: four male members of a single family over three consecutive generations exhibiting anticipation. *British Journal of Haematology*, **100**, 557-560.

Dhingra K, Talpaz M, Riggs, MG, Eastman PS, Zipf T, Ku S & Kurzrock R (1991) Hybridization protection assay: a rapid, sensitive, and specific method for detection of Philadelphia chromosome-positive leukemias. *Blood*, **77**, 238-242.

Dierlamm J, Stul M, Vranckx H, Michaux L, Weghuis DE, Speleman F, Selleslag D, Kramer MH, Noens LA, Cassiman JJ, Van den Berghe H & Hagemeijer A (1998) FISH identifies inv(16)(p13q22) masked by translocations in three cases of acute myeloid leukemia. *Genes Chromosomes and Cancer*, **22**, 87-94.

Dimartino JF & Cleary ML (1999) MLL rearrangements in haematological malignancies: lessons from clinical and biological studies. *British Journal of Haematology,* **106**, 614-626.

Dohner K, Schlenk RF, van der Reijden BA & Dohner H (2000) Deletion of the multidrug resistance-associated protein (MRP1) gene in acute myeloid leukemia with inversion of chromosome 16 has no prognostic impact. *Leukemia*, **14**, 1154.

Downing JR, Head DR, Curcio-Brint AM, Hulshof MG, Motroni TA, Raimondi SC, Carroll AJ, Drabkin HA, Willman C & Theil KS (1993) An AML1/ETO fusion transcript is consistently detected by RNA-based polymerase chain reaction in acute myelogenous leukemia containing the (8;21)(q22;q22) translocation. *Blood*, **81**, 2860-2865.

Downing JR (1999) The AML1-ETO chimaeric transcription factor in acute myeloid leukaemia: biology and clinical significance. *British Journal of Haematology*, **106**, 296-308.

Downing JR, Higuchi M, Lenny N & Yeoh AE-J (2000) Alterations of the AML1 transcription factor in human leukemia. *Seminars in Cell and Developmental Biology*, **11**, 347-360.

Downing JR (2001) The AML1/CBFβ transcription complex: its role in normal haematopoiesis and leukemia. *Leukemia*, **15**, 664-665.

Dowton SB, Beardsley D, Jamison D, Blattner S & Li FP (1985) Studies of a familial platelet disorder. *Blood*, **65**, 557-563.

Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, Capdeville R & Talpaz M (2001a) Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *New England Journal of Medicine*, **344**, 1038-1042.

Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantajian H, Capdeville R, Ohno-Jones S & Sawyers CL (2001b) Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *New England Journal of Medicine*, **344**, 1031-1037.

Eder M, Battmer K, Kafert S, Stucki A, Ganser A & Hertenstein B (1999) Monitoring of BCR-ABL expression using real-time RT-PCR in CML after bone marrow or peripheral blood stem cell transplantation. *Leukemia*, **13**, 1383-1389.

Elmaagacli AH, Beelen DW, Stockova J, Trzensky S, Kroll M, Schaefer UW, Stein C & Opalka B (1997) Detection of AML1/ETO fusion transcripts in patients with t(8;21) acute myeloid leukemia after allogeneic bone marrow transplantation or peripheral blood progenitor cell transplantation. *Blood*, **90**, 3230-3231.

Enright H, Weisdorf D, Peterson L, Rydell RE, Kaplan ME & Arthur DC (1992) Inversion of chromosome 16 and dysplastic eosinophils in accelerated phase of chronic myeloid leukemia. *Leukemia*, **6**, 381-384.

Erickson P, Gao J, Chang KS, Look T, Whisenant E, Raimondi S, Lasher R, Trujillo J, Rowley J & Drabkin H (1992) Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to Drosophila segmentation gene, runt. *Blood*, **80**, 1825-1831.

Erickson PF, Robinson M, Owens G & Drabkin HA (1994) The ETO portion of acute myeloid leukemia t(8;21) fusion transcript encodes a highly evolutionary conserved, putatative transcription factor. *Cancer Research*, **54**, 1782-1786.

Estey EH (2001) Growth factors in acute myeloid leukaemia. *Best Practice and Research Clinical Haematology*, **14**, 175-187.

Evans PA, Short MA, Jack AS, Norfolk DR, Child JA, Shiach CR, Davies F, Tobal K, Liu Yin JA & Morgan GJ (1997) Detection and quantitation of the CBF beta/MYH11 transcripts associated with the inv(16) in presentation and follow-up samples from patients with AML. *Leukemia*, **11**, 364-369.

Faderl S, Talpaz M, Estrov Z, O'Brien S, Kurzrock R & Kantarjian HM (1999a) The biology of chronic myeloid leukemia. *New England Journal of Medicine*, **341**, 164-172.

Faderl S, Talpaz M, Kantarjian HM & Estrov Z (1999b) Should polymerase chain reaction analysis to detect minimal residual disease in patients with chronic myelogenous leukemia be used in clinical decision making? *Blood*, **93**, 2755-2759.

Fearon ER (1997) Human cancer syndromes: clues to the origin and nature of cancer. *Science*, **278**, 1043-1050.

Fenaux P, Preudhomme C, Lai JL, Morel P, Beuscart R & Bauters F (1989) Cytogenetics and their prognostic value in de novo acute myeloid leukaemia: a report on 283 cases. *British Journal of Haematology*, **73**, 61-67.

Fenaux P, Lai JL, Preudhomme C, Jouet JP, Deminatti M & Bauters F (1990) Is translocation (8;21) a 'favorable' cytogenetic rearrangement in acute myeloid leukemia? *Nouvelle Revues Francais Hematologique*, **32**, 179-182.

Fenaux P, Chomienne C & Degos L (1997) Acute promyelocytic leukemia: biology and treatment. *Seminars in Hematology*, **24**, 92-102.

Fenrick R, Amann JM, Lutterbach B, Wang L, Westendorff JJ, Downing JR & Hiebert SW (1999) Both TEL and AML-1 contribute repression domains to the t(12;21) fusion protein. *Molecular and Cellular Biology*, **19**, 6566-6574.

Fero ML, Randel E, Gurley KE, Robert JM & Kemp C (1998) The murine gene p27kip1 is haplo-insufficient for tumour suppression. *Nature*, **396**, 177-180.

Ferrara FF, Fazi F, Bianchini A, Padula F, Gelmetti V, Minucci S, Mancini M, Pelicci PG, Lo Coco F & Nervi C (2001) Histone deacetylase-targeted treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia. *Cancer Research*, **61**, 2-7.

Ferrari T, Weber B, Pils S, Harbott J & Borkhardt A (2001) Absence of point mutations within the AML-1 gene in patients with MDS/AML and loss of chromosome 5q or 7. *Annals of Hematology*, **80**, 72-73.

Fey MF, Pilkington SP, Summers C & Wainscoat JS (1987) Molecular diagnosis of haematological disorders using DNA from stored bone marrow slides. *British Journal of Haematology*, **67**, 489-492.

Fraser JK, Lill MC & Figlin RA (1996) The biology of the cytokine sequence cascade. *Seminars in Hematology*, **23**, 2-8.

Freeman WM, Walker SJ & Vrana KE (1999) Quantitative RT-PCR: pitfalls and potential. *Biotechniques*, **26**, 124-125.

Friedman AD (1999) Leukemogenesis by CBF oncoproteins. *Leukemia*, **13**, 1932-1942.

Fujimaki S, Funato T, Harigae H, Imaizumi M, Suzuki H, Kaneko Y, Miura Y & Sasaki T (2000) A quantitative reverse transcriptase polymerase chain reaction method for the detection of leukemic cells with t(8;21) in peripheral blood. *European Journal of Haematology*, **64**, 252-258.

Gallego M, Carroll AJ, Gad GS, Pappo A, Head D, Behm F, Ravindranath Y & Raimondi SC (1994) Variant t(8;21) rearrangements in acute myeloblastic leukemia of childhood. *Cancer Genetics and Cytogenetics*, **75**, 139-144.

Gamou T, Kitamura E, Hosoda F, Shimizu K, Shinohara K, Hayashi Y, Nagase T, Yokoyama Y & Ohki M (1998) The partner gene of AML1 in t(16;21) myeloid malignancies is a novel member of the MTG8 (ETO) family. *Blood*, **91**, 4028-4037.

Gelmetti V, Zhang J, Fanelli M, Minucci S, Pelicci PG & Lazar MA (1998) Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. *Molecular and Cellular Biology*, **18**, 7185-7191.

Gerrard JM, Israels ED, Bishop AJ, Schroeder ML, Beattie LL, McNicol A, Israels SJ, Walz D, Greenberg AH, Ray M & Israels LG (1991) Inherited platelet-storage pool deficiency associated with a high incidence of acute myeloid leukaemia. *British Journal of Haematology*, **79**, 246-255.

Ghozi MC, Bernstein Y, Negreanu V, Levanon D & Groner Y (1996) Expression of the human acute myeloid leukemia gene AML1 is regulated by two promoter regions. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 1935-1940.

Giles RH, Peters DJ & Breuning MH (1998) Conjunction dysfunctiom: CBP/p300 in human disease. *Trends in Genetics*, **14**, 178-183.

Goldman JM, Gale RP, Horowitz MM, Biggs JC, Champlin RE, Gluckman E, Hoffmann, RG, Jacobsen SJ, Marmont AM, McGlave PB, Messner HA, Rimm AA, Rozman C, Speck B, Tura S, Weiner RS & Bortin MM (1988) Bone marrow transplantation for chronic myelogenous leukemia in chronic phase. Increased risk for relapse associated with T-cell depletion. *Annals of Internal Medicine*, **108**, 806-814.

Greaves MF (1997) Aetiology of acute leukaemia. *Lancet*, **349**, 344-349.

Grignani F, De Matteis S, Nervi C, Tomassoni L, Gelmetti V, Cioce M, Fanelli M, Ruthardt M, Ferrara FF, Zamir I, Seiser C, Grignani F, Lazar MA, Minucci S & Pelicci PG (1998) Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. *Nature*, **391**, 815-818.

Grimwade D, Gorman P, Duprez E, Howe K, Langabeer S, Oliver F, Walker H, Culligan D, Waters J, Pomfret M, Goldstone A, Burnett A, Freemont P, Sheer D & Solomon E (1997) Characterization of cryptic rearrangements and variant translocations in acute promyelocytic leukemia. *Blood*, **90**, 4876-4885.

Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, Rees J, Hann I, Stevens R, Burnett A & Goldstone A (1998) The importance of diagnostic cytogenetics on outcome of AML: analysis of 1612 patients entered into the MRC AML 10 trial. *Blood*, **92**, 2322-2333.

Gundestrup M & Storm HH (1999) Radiation-induced acute myeloid leukaemia and other cancers in commercial jet cockpit crew: a population-based cohort study. *Lancet*, **354**, 2029-2031.

Gustincich S, Manfioletti G, Del Sal G, Schneider C & Carninci P (1991) A fast method for high-quality genomic DNA extraction from whole human blood. *Biotechniques*, **11**, 298-302.

Hackwell SM, Robinson DO, Harvey JF & Ross FM (1999) Identification of false-positive CBFbeta/MYH11 RT-PCR results. *Leukemia*, **13**, 1617-1619.

Haferlach T (2001) Quantitative reverse transcriptase-polymerase chain reaction in AML with t(8;21)(q22;q22). *Leukemia Research*, **25**, 55-56.

Hagemeijer A, Garson OM & Kondo K (1984) Fourth International Workshop on Chromosomes in Leukemia 1982: Translocation (8;21)(q22;q22) in acute nonlymphocytic leukemia. *Cancer Genetics and Cytogenetics*, **11**, 284-287.

Hagemeijer A, de Klein A, Wijsman J, van Meerten E, de Greef GE & Sacchi N (1998) Development of an interphase fluorescent in situ hybridization (FISH) test to detect t(8;21) in AML patients. *Leukemia*, **12**, 96-101.

Hall PD, Virella G, Willoughby T, Atchley DH, Kreitman RJ & Frankel AE (2001) Antibody response to DT-GM, a novel fusion toxin consisting of a truncated diphtheria toxin (DT) linked to human granulocyte-macrophage colony stimulating factor (GM), during a phase I trial of patients with relapsed or refractory acute myeloid leukemia. *Clinical Immunology*, **100**, 191-197.

Hann IM, Stevens RF, Goldstone AH, Rees JKH., Wheatley K, Gray RG & Burnett AK (1997) Randomised comparison of DAT versus ADE as induction chemotherapy in children and younger adults with acute myeloid leukemia. Results of the Medical Research Council's 10th AML trial (MRC AML 10). *Blood*, **89**, 2311-2318.

Hara T & Miyajima A (1999) Cytokine receptors and signal transduction. In: *Textbook of malignant haematology*. Eds: Degos L, Linch DC & Lowenberg B, Martin Dunitz, London.

Harada Y, Harada H, Downing JR & Kimura A (2001) A hematopoietic-specific transmembrane protein, Art-1, is possibly regulated by AML1. *Biochemical and Biophysical Research Communications*, **284**, 714-722.

Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, Lister TA & Bloomfield CD (1999) World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting - Airlie House, Virginia, November 1997. *Journal of Clinical Oncology*, **17**, 3835-3849.

Harrison CJ, Radford-Weiss I, Ross F, Rack K, le Guyader G, Vekemans M & Macintyre E (1999) Fluorescence in situ hybridization analysis of masked (8;21)(q22;q22) translocations. *Cancer Genetics and Cytogenetics*, **112**, 15-20.

Hattori M, Fujiyama A, Taylor TD, Watanabe H, Yada T, Park H-S, Toyoda A, Ishii K, Totoki Y, Choi D-K, Soeda E, Ohki M, Takagi T, Sakaki Y, Taudien S, Blechscmidt K, Polley A, Menzel U, Delabar J, Kumpf K, Lehmann R, Patterson D, Reichwald K, Rump A, Schillhabel M, Schudy A, Zimmermann W, Rosenthal A, Kudoh J, Shibuya K, Kawasaki K, Asakawa S, Shintani A, Sasaki T, Nagamine K, Mitsuyama S, Antonarakis SE, Minoshima S, Shimizu M, Nordsiek G, Hornischer K, Brandt P, Scharfe M, Schon O, Desario A, Reichelt J, Kauer G, Blocker H, Ramser J, Beck A, Klages S, Hennig S, Riesselmann L, Dagand E, Haaf T, Wehrmeyer S, Borzym K, Gardiner K, Nizetic, D, Francis F, Lehrach H, Reinhardt R & Yaspo M-L (2000) The DNA sequence of chromosome 21. *Nature*, **405**, 311-319.

He LZ, Tribioli C, Rivi R, Peruzzi D, Pelicci PG, Soares V, Cattoretti G & Pandolfi PP (1997) Acute leukemia with promyelocytic features in PML/RARalpha mice. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 5302-5307.

Hebert J, Cayuela JM, Daniel MT, Berger R & Sigaux F (1994) Detection of minimal residual disease in acute myelomonocytic leukemia with abnormal marrow eosinophils by nested polymerase chain reaction with allele specific amplification. *Blood*, **84**, 2291-2296.

Heim S & Mitelman F (1995) Acute myeloid leukemia. *Cancer Cytogenetics: Chromosomal and molecular genetic aberrations of tumor cells*, 69-140. Wiley-Liss, New York.

Heisterkamp N, Jenster G, ten Hoeve J, Zovich D, Pattengale PK & Groffen J (1990) Acute leukemia in bcr/abl transgenic mice. *Nature*, **344**, 251-253.

Hernandez A, Osterholz J, Price CM, Wiedemann LM, Gordon MY, Goldman JM & Morgan GJ (1990) Detection of the hybrid BCR/ABL messenger RNA in single CFU-GM colonies using the polymerase chain reaction. *Experimental Hematology*, **18**, 1142-1144.

Hiebert SW, Sun W, Davis JN, Golub T, Shurtleff SA, Buijs A, Downing JR, Grosveld G, Roussell MF, Gilliland DG, Lenny N & Meyers S (1996) The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. *Molecular and Cellular Biology*, **16**, 1349-1355.

Higuchi M, O'Brien D, Lenny N, Yang S, Cai Z & Downing JR (2000) Expression of AML1-ETO immortalizes myeloid progenitors and cooperates with secondary mutations to induce granulocytic sarcoma/acute myeloid leukemia. *Blood*, **96**, 222a.

Hiorns LR, Min T, Swansbury GJ, Zelent A, Dyer MJ & Catovsky D (1994) Interstitial insertion of retinoic acid receptor-alpha gene in acute promyelocytic leukemia with normal chromosomes 15 and 17. *Blood*, **83**, 2946-2951.

Ho CY, Otterud B, Legare RD, Varvil T, Saxena R, DeHart DB, Kohler SE, Aster JC, Dowton SB, Li FP, Leppert M & Gilliland DG (1996) Linkage of a familial platelet disorder with a propensity to develop myeloid malignancies to human chromosome 21q22.1-22.2. *Blood*, **87**, 5218-5224.

Hokland P & Pallisgaard N (2000) Integration of molecular methods for detection of balanced translocations in the diagnosis and follow-up of patients with leukemia. *Seminars in Hematology*, **37**, 358-367.

Horwitz M, Sabath DE, Smithson WA & Radich J (1996a) A family inheriting different subtypes of acute myelogenous leukemia. *American Journal of Hematology*, **52**, 295-304.

Horwitz M, Goode EL & Jarvik GP (1996b) Anticipation in familial leukemia. *American Journal of Human Genetics*, **59**, 990-998.

Horwitz M (1997) The genetics of familial leukemia. *Leukemia*, **11**, 1347-1359.

Horwitz M, Benson KF, Li F-Q, Wolff J, Leppert MF, Hobson L, Mangelsdorf M, Yu S, Hewett D, Richards RI & Raskind WH (1997) Genetic heterogeneity in familial acute myelogenous leukemia: evidence for a second locus at chromosome 16q21-23.2. *American Journal of Human Genetics*, **61**, 873-881.

Hromas R, Shopnick R, Jumean HG, Bowers C, Varella-Garcia M & Richkind K (2000) A novel syndrome of radiation-associated acute myeloid leukemia involving AML1 gene translocations. *Blood*, **95**, 4011-4013.

Huang X, Crute BE, Sun C, Tang YY, Kelley JJ, Lewis AF, Hartman KL, Laue TM, Speck NA & Bushweller JH (1998) Overexpression, purification, and biophysical characterization of the heterodimerization domain of the core-binding factor beta subunit. *Journal of Biological Chemistry*, **273**, 2480-2487.

Huang X, Peng JW, Speck NA & Bushweller JH (1999) Solution structure of core binding factor β and map of the CBFα binding site. *Nature Structural Biology*, **6**, 624-627.

Huang G, Shigesada K, Ito K, Wee HJ, Yokomizo T & Ito Y (2001) Dimerization with PEBP2beta protects RUNX1/AML1 from ubiquitin-proteasome-mediated degradation. *EMBO Journal*, **20**, 723-733.

Huntington's Disease Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell*, **72**, 971-983.

Imai Y, Kurokawa M, Izutsu K, Hangaishi A, Takeuchi K, Maki K, Ogawa S, Chiba S, Mitani K & Hirai H (2000) Mutations of the AML1 gene in myelodysplastic syndromes and their functional implications in leukemogenesis. *Blood*, **96**, 3154-3160.

Johannson B, Mertens F & Mitelman F (1994) Secondary chromosomal abnormalities in acute leukemia. *Leukemia*, **8**, 953-962.

Jurlander J, Caligiuri MA, Ruutu T, Baer MR, Strout MP, Oberkircher AR, Hoffmann L, Ball ED, Frei-Lahr DA, Christiansen NP, Block AM, Knuutila S, Herzig GP & Bloomfield CD (1996) Persistence of the AML1/ETO fusion transcript in patients treated with allogeneic bone marrow transplantation for t(8;21) leukemia. *Blood*, **88**, 2183-2191.

Kagoshima H, Akamatsu Y, Ito Y & Shigesada K (1996) Functional dissection of the alpha and beta subunits of transcription factor PEBP2 and the redox susceptibility of its DNA binding activity. *Journal of Biological Chemistry*, **271**, 33074-33082.

Kamachi Y, Ogawa E, Asano M, Ishida S, Murakami Y, Satake M, Ito Y & Shigesada K (1990) Purification of a mouse nuclear factor that binds to both the A and B cores of the polyoma ehancer. *Journal of Virology*, **64**, 4808-4819.

Kanno Y, Kanno T, Sakakura C, Bae SC & Ito Y (1998) Cytoplasmic sequestration of the polyomavirus enhancer binding protein 2 (PEBP2)/core binding factor alpha (CBFalpha) subunit by the leukemia-related PEBP2/CBF beta-SMMHC fusion protein inhibits PEBP2/CBF-mediated transactivation. *Molecular and Cellular Biology*, **18**, 4252-4261.

Kantarjian HM, Keating MJ, Walters RS, Smith TL, Cork A, McCredie KB & Freireich EJ (1986) Therapy-related leukemia and myelodysplastic syndrome: clinical, cytogenetic, and prognostic features. *Journal of Clinical Oncology*, **4**, 1748-1757.

Keating MJ, Smith TL, Kantarjian H, Cork A, Walters R, Trujillo JM, McCredie KB, Gehan EA & Freireich EJ (1988) Cytogenetic pattern in acute myelogenous leukemia: a major reproducible determinant of outcome. *Leukemia*, **2**, 403-412.

Kim MH, Stewart J, Devlin C, Kim YT, Boyd E & Connor M (2001) The application of comparative genomic hybridization as an additional tool in the chromosome analysis of acute myeloid leukemia and myelodysplastic syndromes. *Cancer Genetics and Cytogenetics*, **126**, 26-33.

Kinzler KW & Vogelstein B (1996) Lessons from hereditary colorectal cancer. *Cell*, **87**, 159-170.

Kitabayashi I, Yokoyama A, Shimizu K & Ohki M (1998a) Interaction and functional cooperation of the leukemia-associated factors AML1 and p300 in myeloid cell differentiation. *EMBO Journal*, **17**, 2994-3004.

Kitabayashi I, Ida K, Morohoshi F, Yokoyama A, Mitsuhashi N, Shimizu K, Nomura N, Hayashi Y & Ohki M (1998b) The AML1-MTG8 leukemic fusion protein forms a complex with a novel member of the MTG8 (ETO/CDR) family, MTGR1. *Molecular and Cellular Biology,* **18**, 846-858.

Kitamura T, Tange T, Terasawa T, Chiba S, Kuwaki T, Miyagawa K, Piao YF, Miyazono K, Urabe A & Takaku F (1989) Establishment and characterization of a unique human cell line that proliferates dependently of GM-CSF, IL-3, or erythropoietin. *Journal of Cell Physiology*, **140**, 323-334.

Komori T, Yagi H, Nomura S, Yamaguchi S, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S Kishimoto T (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*, **89**, 755-764.

Komori T & Kishimoto T (1998) Cbfa1 in bone development. *Current Opinion in Genetics and Development*, **8**, 494-499.

Komuro H, Valentine MB, Rubnitz JE, Saito M, Raimondi SC, Carroll AJ, Yi T, Sheerr CJ & Look AT (1999) p27kip1 deletions in childhood acute lymphoblastic leukemia. *Neoplasia*, **1**, 253-261.

Kondo M, Kudo K, Kimura H, Inaba J, Kato K, Kojima S, Matsuyama T & Horibe K (2000) Real-time reverse transcription-polymerase chain reaction for the detection of AML1-MTG8 fusion transcripts in t(8;21)-positive acute myelogenous leukemia. *Leukemia Research*, **24**, 951-956.

Kosary CL, Ries LG, Miller BA, Hankey BF & Edwards BF (1995) SEER cancer statistics review, 1973-1992: tables and graphs, National Cancer Institute. NIH publication no. 96-96-2789.

Kosugi H, Towatari M, Hatano S, Kitamura K, Kiyoi H, Kinoshita T, Tanimoto M, Murate T, Kawashima K, Saito H & Naoe T (1999) Histone deacetylase inhibitors are the potent inducer/enhancer of differentiation in acute myeloid leukemia: a new approach to anti-leukemia therapy. *Leukemia*, **13**, 1316-1324.

Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, Walker H, Wheatley K, Bowen DT, Burnett AK, Goldstone AH & Linch DC (2001) The presence of a *FLT3* internal tandem duplication in patients with acute myeloid leukemia adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: Analysis of 854 patients from the UK MRC AML 10 and 12 trials. *Blood*, **98**, 1752-1759.

Kozu T, Miyoshi H, Shimizu K, Maseki N, Kaneko Y, Asou H, Kamada N & Ohki M (1993) Junctions of the AML1/MTG8(ETO) fusion are constant in t(8;21) acute myeloid leukemia detected by reverse transcription polymerase chain reaction. *Blood*, **82**, 1270-1276.

Krauter J, Peter W, Paschenberg U, Heinze B, Bergmann L, Hoelzer D, Lubbert M, Schlimok G, Arnold R, Kirchner H, Port M, Ganser A & Heil G (1998) Detection of karyotypic aberrations in acute myeloblastic leukaemia: a prospective comparison between PCR/FISH and standard cytogenetics in 140 patients with de novo AML. *British Journal of Haematology*, **103**, 72-78.

Krauter J, Wattjes MP, Nagel S, Heidenreich O, Krug U, Kafert S, Bunjes D, Bergmann L, Ganser A & Heil G (1999) Real-time RT-PCR for the detection and quantification of AML1/MTG8 fusion transcripts in t(8;21)-positive AML patients. *British Journal of Haematology*, **107**, 80-85.

Kremer EJ, Pritchard M, Lynch M, Yu S, Holman K, Baker E, Warren ST, Schlessinger D, Sutherland GR & Richards RI (1991) Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence. *Science*, **252**, 1711-1714.

Kurokawa M, Tanaka T, Tanaka K, Hirano N, Ogawa S, Mitani K, Yazaki Y & Hirai H (1996) A conserved cysteine residue in the runt homology domain of AML1 is required for the DNA binding ability and the transforming activity on fibroblasts. *Journal of Biological Chemistry*, **271**, 16870-16876.

Kurokawa M, Mitani K, Imai Y, Ogawa S, Yazaki Y & Hirai H (1998) The t(3;21) fusion product, AML1/EVI-1, interacts with Smad3 and blocks transforming growth factor-beta-mediated growth inhibition of myeloid cells. *Blood*, **92**, 4003-4012.

Kurzrock R, Estrov Z, Kantarjian H & Talpaz M (1998) Conversion of interferon-induced, long-term cytogenetic remissions in chronic myelogenous leukemia to polymerase chain reaction negativity. *Journal of Clinical Oncology*, **16**, 1526-1531.

Kusec R, Laczika K, Knobl P, Friedl J, Greinix H, Kahls P, Linkesch W, Schwarzinger I, Mitterbauer G & Purtscher B (1994) AML1/ETO fusion mRNA can be detected in remission blood samples of all patients with t(8;21) acute myeloid leukemia after chemotherapy or autologous bone marrow transplantation. *Leukemia*, **8**, 735-739.

Kuss BJ, Deeley RG, Cole SP, Willman CL, Kopecky KJ, Wolman SR, Eyre HJ, Lane SA, Nancarrow JK & Whitmore SA (1994) Deletion of gene for multidrug resistance in acute myeloid leukaemia with inversion in chromosome 16: prognostic implications. *Lancet*, **343**, 1531-1534.

Laczika K, Novak M, Hilgarth B, Mitterbauer M, Mitterbauer G, Scheidel-Petrovic A, Scholten C, Thalhammer-Scherrer R, Brugger S, Keil F, Schwarzinger I, Haas OA, Lechner K & Jaeger U (1998) Competitive CBFbeta/MYH11 reverse-transcriptase polymerase chain reaction for quantitative assessment of minimal residual disease during postremission therapy in acute myeloid leukemia with inversion(16): a pilot study. *Journal of Clinical Oncology*, **16**, 1519-1525.

Lafage-Pochitaloff M, Alcalay M, Brunel V, Longo L, Sainty D, Simonetti J, Birg F & Pelicci PG (1995) Acute promyelocytic leukemia cases with nonreciprocal PML/RARa or RARa/PML fusion genes. *Blood*, **85**, 1169-1174.

Largaespada DA (2001) Haploinsufficiency for tumor suppression: the hazards of being single and living a long time. *Journal of Experimental Medicine*, **193**, 15-18.

Larson RA, Williams SF, Le Beau MM, Bitter MA, Vardiman JW & Rowley JD (1986) Acute myelomonocytic leukemia with abnormal eosinophils and inv(16) or t(16;16) has a favorable prognosis. *Blood*, **68**, 1242-1249.

Larson RA, Wang Y, Banerjee M, Wiemels J, Hartford C, Le Beau MM & Smith MT (1999) Prevalence of the activating 609C-T polymorphism in the NAD(P)H:quinone oxidoreductase (NQO1) gene in patients with primary and therapy-related myeloid leukemia. *Blood*, **94**, 803-807.

La Spada AR, Wilson EM, Lubahn DB, Harding AE & Fishbeck KH (1991) Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature*, **352**, 77-79.

Le XF, Groner Y, Kornblau SM, Gu Y, Hittelman WN, Levanon D, Mehta K, Arlinghaus RB & Chang KS (1999) Regulation of AML2/CBFA3 in hematopoietic cells through the retinoic acid receptor alpha-dependent signaling pathway. *Journal of Biological Chemistry*, **30**, 21651-21658.

Le Beau MM, Larson RA, Bitter MA, Vardiman JW, Golomb HM & Rowley JD (1983) Association of an inversion of chromosome 16 with abnormal bone marrow eosinophils in acute myelomonocytic leukemia. A unique cytogenetic-clinicopathological association. *New England Journal of Medicine*, **309**, 630-636.

LeBlanc T & Berger R (1997) Molecular cytogenetics of childhood acute myelogenous leukaemias. *European Journal of Haematology*, **59**, 1-13.

Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, Hecht J, Geoffrey V, Ducy P & Karsenty G (1997) Missense mutations abolishing DNA binding of the ostoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. *Nature Genetics*, **16**, 307-310.

Legare RD, Lu D, Gallagher M, Ho C, Tan X, Barker G, Shimizu K, Ohki M, Lenny S, Hiebert S & Gilliland DG (1997) CBFA2, frequently rearranged in leukemia, is not responsible for a familial leukemia syndrome. *Leukemia*, **11**, 2111-2119.

Lenny N, Meyers S & Hiebert SW (1995) Functional domains of the t(8;21) fusion protein, AML-1/ETO. *Oncogene*, **11**, 1761-1769.

Levanon D, Negreanu V, Bernstein Y, Bar-Am I, Avivi L & Groner Y (1994) AML1, AML2, and AML3, the human members of the runt domain gene-family: cDNA structure, expression, and chromosomal localization. *Genomics*, **23**, 425-432.

Levanon D, Bernstein Y, Negreanu V, Ghozi MC, Bar-AM I, Aloya R, Goldenberg D, Lotem J & Groner Y (1996) A large variety of alternatively spliced and differentially expressed mRNAs are encoded by the human acute myeloid leukemia gene AML1. *DNA and Cell Biology*, **15**, 175-185.

Levanon D, Eisenstein M & Groner Y (1998) Site-directed mutagenesis supports a three-dimensional model of the runt domain. *Journal of Molecular Biology*, **277**, 509-512.

Levanon D, Glusman G, Bangsow T, Ben-Asher E, Male DA, Avidan N, Bangsow C, Hattori M, Taylor TD, Taudien S, Blechschmidt K, Shimizu N, Rosenthal A, Sakaki Y, Lancet D & Groner Y (2001) Architecture and anatomy of the genomic locus encoding the human leukemia-associated transcription factor RUNX1/AML1. *Gene*, **262**, 23-33.

Lewin B (2000) Nuclear splicing. In: *Genes VII*, Oxford, Oxford University Press, 685-718.

Licht JD (2001) AML1 and the AML1-ETO fusion protein in the pathogenesis of t(8;21) AML. *Oncogene*, **20**, 5660-5679.

Lin F, van Rhee F, Goldman JM & Cross NC (1996) Kinetics of increasing BCR-ABL transcript numbers in chronic myeloid leukemia patients who relapse after bone marrow transplantation. *Blood*, **87**, 4473-4478.

Lin RJ, Nagy L, Inoue S, Shao W, Miller WH & Evans RM (1998) Role of histone deacetylase complex in acute promyelocytic leukemia. *Nature*, **391**, 811-814.

Lion T, Henn T, Gaiger A, Kalhs P & Gadner H (1993) Early detection of relapse after bone marrow transplantation in patients with chronic myelogenous leukaemia. *Lancet*, **341**, 275-276.

Lion T (1996) Appropriate control genes for RT-PCR. *Leukemia*, **10**, 1843.

Lion T (2001) Current recommendations for positive controls in RT-PCR. *Leukemia*, **15**, 1033-1037.

Litmanovich D, Zamir-Brill R, Jeison M & Gershoni-Baruch R (2000) Is inversion 16 a prerequisite and is trisomy 22 invariably associated with inversion 16 in AML-M4eo? *Cancer Genetics and Cytogenetics*, **121**, 106.

Liu P, Claxton DF, Marlton P, Hajra A, Siciliano J, Freedman M, Chandrasekharappa SC, Yanagisaw, K, Stallings RL & Collins FS (1993a) Identification of yeast artificial chromosomes containing the inversion 16 p-arm breakpoint associated with acute myelomonocytic leukemia. *Blood*, **82**, 716-721.

Liu P, Tarle SA, Hajra A, Claxton DF, Marlton P, Freedman M, Siciliano MJ & Collins FS (1993b) Fusion between transcription factor CBFbeta/PEBP2 beta and a myosin heavy chain in acute myeloid leukemia. *Science*, **261**, 1041-1044.

Liu PP, Hajra A, Wijmenga C & Collins FS (1995) Molecular pathogenesis of the chromosome 16 inversion in the M4E0 subtype of acute myeloid leukemia. *Blood*, **85**, 2289-2302.

Liu PP, Wijmenga C, Hajra A, Blake TB, Kelley CA, Adelstein RS, Bagg A, Rector J, Cotelingam J, Willman CL & Collins FS (1996) Identification of the chimeric protein product of the CBFB-MYH11 fusion gene in inv(16) leukemia cells. *Genes Chromosomes and Cancer*, **16**, 77-87.

Look AT (1997) Oncogenic transcription factors in the human acute leukemias. *Science*, **278**, 1059-1064.

Lowenberg B, Downing JR & Burnett A (1999) Acute myeloid leukemia. *New England Journal of Medicine*, **341**, 1051-1062.

Luddy RE, Champion LAA & Schwartz AD (1978) A fatal myeloproliferative syndrome in a family with thrombocytopenia and platelet dysfunction. *Cancer*, **41**, 1959-1963.

Lutterbach B, Westendorf JJ, Linggi B, Patten A, Moniwa M, Davie JR, Huynh KD, Lavinsky RM, Rosenfeld MG, Glass C, Seto E & Hiebert SW (1998) ETO, a target of t(8;21) in acute leukemia, interacts with the N-CoR and mSin3 copressors. *Molecular and Cellular Biology*, **18**, 7176-7184.
Lutterbach B & Hiebert SW (2000) Role of the transcription factor AML-1 in acute leukemia and hematopoietic differentiation. *Gene*, **245**, 223-235.

Mackinnon S, Papadopoulos EB, Carabasi MH, Reich L, Collins NH, Boulad F, Castro-Malaspina H, Childs BH, Gillio AP & Kernan NA (1995) Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood*, **86**, 1261-1268.

Mancini M, Cedrone M, Diverio D, Emanuel B, Stul M, Vranckx H, Brama M, De Cuia MR, Nanni M, Fazi F, Mecucci C, Alimena G & Hagemeijer A (2000) Use of dual-color interphase FISH for the detection of inv(16) in acute myeloid leukemia at diagnosis, relapse and during follow-up: a study of 23 patients. *Leukemia*, **14**, 364-368.

Mandelli F, Petti MC, Lo Coco F (1998) Therapy of acute myeloid leukemia: towards a patient-oriented, risk-adapted approach. *Haematologica*, **83**, 1015-1023.

Marcucci G, Caligiuri MA & Bloomfield CD (1997) Defining the 'absence' of the CBFbeta/MYH11 fusion transcript in patients with acute myeloid leukemia and inversion of chromosome 16 to predict long-term complete remission: a call for definitions. *Blood*, **90**, 5022-5024.

Marcucci G, Livak KJ, Bi W, Strout MP, Bloomfield CD & Caligiuri MA (1998) Detection of minimal residual disease in patients with AML1/ETO-associated acute myeloid leukemia using a novel quantitative reverse transcription polymerase chain reaction assay. *Leukemia*, **12**, 1482-1489.

Marcucci G, Caligiuri MA, Dohner H, Archer KJ, Schlenk RF, Dohner K, Maghraby EA & Bloomfield CD (2001) Quantification of CBFbeta/MYH11 fusion transcripts by real time RT-PCR in patients with inv(16) acute myeloid leukemia. *Leukemia*, **15**, 1072-1080.

Marlton P, Claxton DF, Liu P, Estey EH, Beran M, LeBeau M, Testa JR, Collins FS, Rowley JD & Siciliano MJ (1995) Molecular characterization of 16p deletions associated with inversion 16 defines the critical fusion for leukemogenesis. *Blood*, **85**, 772-779.

Marlton P, Keating M, Kantarjian H, Pierce S, O'Brien S, Freireich EJ & Estey E (1995) Cytogenetic and clinical correlates in AML patients with abnormalities of chromosome 16. *Leukemia*, **9**, 965-971.

Martinelli G, Ottaviani E, Testoni N, Montefusco V, Pastano R & Tura S (2000) Long-term disease-free acute myeloblastic leukemia with inv(16) is associated with PCR undetectable CBFβ/MYH11 transcripts. *Haematologica*, **85**, 552-555.

Martinet D, Muhlematter D, Leeman M, Parlier V, Hess U, Gmur J & Jotterand M (1997) Detection of 16p deletions by FISH in patients with inv(16) or t(16;16) and acute myeloid leukemia (AML). *Leukemia*, **11**, 964-970.

Martinez-Climent JA, Comes AM, Vizcarra E, Reshmi S, Benet I, Marugan I, Tormo M, Terol MJ, Solano C, Arbona C, Prosper F, Barragan E, Bolufer P, Rowley JD & Garcia-Conde J (1999) Variant three-way translocation of inversion 16 in AML-M4Eo confirmed by fluorescence in situ hybridization analysis. *Cancer Genetics and Cytogenetics*, **110**, 111-114.

Maruyama F, Yang P, Stass SA, Cork A, Freireich EJ, Lee MS & Chang KS (1993) Detection of the AML1/ETO fusion transcript in the t(8;21) masked translocation in acute myelogenous leukemia. *Cancer Research*, **53**, 4449-4451.

Maseki N, Miyoshi H, Shimizu K, Homma C, Ohki M, Sakurai M & Kaneko Y (1993) The 8;21 chromosome translocation in acute myeloid leukemia is always detectable by molecular analysis using AML1. *Blood*, **81**, 1573-1579.

Matsushita H, Kizaki M, Kobayashi H, Muto A & Ikeda Y (1999) Induction of apoptosis in myeloid leukaemic cells by ribozymes targeted against AML1/MTG8. *British Journal of Cancer*, **79**, 1325-1331.

Medvinsky A & Dzierak E (1996) Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell*, **86**, 897-906.

Medvinsky A & Dzierak E (1999) Development of the hematopoietic stem cell: can we describe it? *Blood*, **94**, 3613-3614.

Mehta AB, Bain BJ, Fitchett M, Shah S & Secker-Walker L (1998) Trisomy 13 and myeloid malignancy: characteristic blast cell morphology. A United Kingdom Cancer Cytogenetics Group survey. *British Journal of Haematology*, **101**, 749-752.

Melo JV (1997) BCR-ABL gene variants. *Baillieres Clinical Haematology*, **10**, 203-222.

Mensink E, van de Locht A, Schattenberg A, Linders E, Schaap N, Geurts van Kessel A & De Witte T (1998) Quantitation of minimal residual disease in Philadelphia chromosome positive chronic myeloid leukaemia patients using real-time quantitative RT-PCR. *British Journal of Haematology*, **102**, 768-774.

Metcalf D (1998) Regulatory mechanisms controlling hematopoiesis: principles and problems. *Stem Cells*, **16**, 3-11.

Meyers S, Downing JR & Hiebert SW (1993) Identification of AML-1 and the (8;21) translocation protein (AML-1/ETO) as sequence-specific DNA-binding proteins: the runt homology domain is required for DNA binding and protein-protein interactions. *Molecular and Cell Biology*, **13**, 6336-6345.

Meyers S, Lenny N & Hiebert S (1995) The t(8;21) fusion protein interferes with AML-1B dependent transcriptional activation. *Molecular and Cell Biology*, **15**, 1974-1982.

Mitelman F (1995) ISCN: An international system for human cytogenetic nomenclature. *S.Karger*, Basel, Switzerland.

Mitterbauer M, Kusec R, Schwarzinger I, Haas OA, Lechner K & Jaeger U (1998) Comparison of karyotype analysis and RT-PCR for AML1/ETO in 204 unselected patients with AML. *Annals of Hematology*, **76**, 139-143.

Mitterbauer M, Laczika K, Novak M, Mitterbauer G, Hilgarth B, Pirc-Danoewinata H, Schwarzinger I, Haas OA, Fonatsch C, Lechner K & Jaeger U (2000) High concordance of karyotype analysis and RT-PCR for CBF beta/MYH11 in unselected patients with acute myeloid leukemia. A single center study. *American Journal of Clinical Pathology*, **113**, 406-410.

Miyamoto T, Nagafuji K, Harada M, Eto T, Fujisaki T, Kubota A, Akashi K, Mizuno S, Takenaka K & Kanaji T (1995) Quantitative analysis of AML1/ETO transcripts in peripheral blood stem cell harvests from patients with t(8;21) acute myelogenous leukaemia. *British Journal of Haematology*, **91**, 132-138.

Miyamoto T, Nagafuji K, Akashi K, Harada M, Kyo T, Akashi T, Takenaka K, Mizuno S, Gondo H, Okamura T, Dohy H & Niho Y (1996) Persistence of multipotent progenitors expressing AML1/ETO transcripts in long-term remission patients with t(8;21) acute myelogenous leukemia. *Blood*, **87**, 4789-4796.

Miyamoto T, Weissman IL & Akasi K (2000) AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 7521-7526.

Miyoshi H, Shimizu K, Kozu T, Maseki N, Kaneko Y & Ohki M (1991) t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 10431-10434.

Miyoshi H, Kozu T, Shimizu K, Enomoto K, Maseki N, Kaneko Y, Kamada N & Ohki M (1993) The t(8;21) translocation in acute myeloid leukemia results in production of an AML1-MTG8 fusion transcript. *EMBO Journal*, **12**, 2715-2721.

Miyoshi H, Ohira M, Shimizu K, Mitani K, Hirai H, Imai T, Yokoyama K, Soeda E & Ohki M (1995) Alternative splicing and genomic structure of the AML1 gene involved in acute myeloid leukemia. *Nucleic Acids Research*, **23**, 2762-2769.

Mohr B, Bornhauser M, Thiede C, Schakel U, Schaich M, Illmer T, Paschenberg U & Ehninger G (2000) Comparison of spectral karyotyping and conventional cytogenetics in 39 patients with acute myeloid leukemia and myelodysplastic syndrome. *Leukemia*, **14**, 1031-1038.

Monahan BP, Rector JT, Liu PP, Cotelingham JD & Dahut W (1996) Clinical aspects of expression of inversion 16 chromosomal fusion transcript CBFB/MYH11 in acute myelogenous leukemia subtype M1 with abnormal bone marrow eosinophilia. *Leukemia*, **10**, 1653-1654.

Morin MJ (2000) From oncogene to drug: development of small molecule tyrosine kinase inhibitors as anti-tumour an anti-angiogenic agents. *Oncogene*, **19**, 6574-6583.

Morschhauser F, Cayuela JM, Martini S, Baruchel A, Rousselot P, Socie G, Berthou P, Jouet JP, Straetmans N, Sigaux F, Fenaux P & Preudhomme C (2000) Evaluation of minimal residual disease using reverse-transcription polymerase chain reaction in t(8;21) acute myeloid leukemia: a multicenter study of 51 patients. *Journal of Clinical Oncology*, **18**, 788-794.

Mrozek K, Heinonen K & Bloomfield CD (2001a) Clinical importance of cytogenetics in acute myeloid leukaemia. *Best Practice and Research Clinical Haematology*, **14**, 19-47.

Mrozek K, Prior TW, Edwards C, Marcucci G, Carroll AJ, Snyder PJ, Koduru PR. Theil KS, Pettenati MJ, Archer KJ, Caligiuri MA, Vardiman JW, Kolitz JE, Larson RA & Bloomfield CD (2001b) Comparison of cytogenetics and molecular genetic detection of t(8;21) and inv(16) in a prospective series of adults with de novo acute myeloid leukemia: a cancer and leukemia group B study. *Journal of Clinical Oncology*, **19**, 2482-2492.

Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, Lindhout D, Cole WG, Henn W, Knoll JH, Owen MJ, Mertelsmann R, Zabel BU & Olsen BR (1997) Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell*, **89**, 773-779.

Muto A, Mori S, Matsushita H, Awaya N, Ueno H, Takayama N, Okamoto S, Kizaki M & Ikeda Y (1996) Serial quantification of minimal residual disease of t(8;21) acute myelogenous leukaemia with RT-competitive PCR assay. *British Journal of Haematology*, **95**, 85-94.

Nagafuchi S, Yanagisawa H, Ohsaki E, Shirayama T, Tadokoro K, Inoue T & Yamada M (1994) Structure and expression of the gene responsible for the triplet repeat disorder, dentatorubral and pallidoluysiar atrophy (DRPLA). *Nature Genetics*, **8**, 177-182.

Nagata T, Gupta V, Sorce D, Kim WY, Sali A, Chait BT, Shigesada K, Ito Y & Werner MH (1999) Immunoglobulin motif DNA recognition and heterodimerization of the PEBP2/CBF Runt domain. *Nature Structural Biology*, **6**, 615-619.

Nagata T & Werner MH (2001) Functional mutagenesis of AML1/RUNX1 and PEBP2β/CBFβ define distinct, non-overlapping sites for DNA recognition and heterodimerization by the runt domain. *Journal of Molecular Biology*, **308**, 191-203.

Narayanan MN, Geary CG, Harrison CJ, Cinkotai KI & Lewis MJ (1993) Three cases of the myelodysplastic syndrome with pericentric inversion of chromosome 16. *British Journal of Haematology*, **85**, 217-219.

Nelson NC (1998) Rapid detection of genetic mutations using the chemiluminescent hybridization protection assay (HPA): overview and comparison with other methods. *Critical Revues of Clinical Laboratory Science*, **35**, 369-414.

Niedobitek G, Meru N & Delecluse HJ (2001) Epstein-Barr virus infection and human malignancies. *International Journal of Experimental Pathology*, **82**, 149-170.

Nisson PE, Watkins PC & Sacchi N (1992) Transcriptionally active chimeric gene derived from the fusion of the AML1 gene and a novel gene on chromosome 8 in t(8;21) leukemic cells. *Cancer Genetics and Cytogenetics*, **63**, 81-88.

Novak M, Laczika K, Mitterbauer M, Purtscher B, Scheidel-Petrovic A, Lechner K, Jaeger U & Haas OA (1995) Novel CBF beta-MYH11 fusion transcripts and alternative splicing in acute myeloid leukemia with inversion of chromosome 16. *Blood*, **86**, 2449-2450.

Nowell PC & Hungerford DA (1960) A minute chromosome in human chronic granulocytic leukemia. *Science*, **132**, 1497-1502.

Nuchprayoon I, Meyers S, Scott LM, Suzow J, Hiebert S & Freidman AD (1994) PEBP2/CBF, the murine homolog of the human AML1 and PEBP2β/CBFβ proto-oncoproteins, regulates the murine myeloperoxidase and neutrophil elastase genes in immature myeloid cells. *Molecular and Cellular Biology*, **14**, 5558-5568.

Nucifora G, Birn DJ, Erickson P, Gao J, LeBeau MM, Drabkin HA & Rowley JD (1993a) Detection of DNA rearrangements in the AML1 and ETO loci and of an AML1/ETO fusion mRNA in patients with t(8;21) acute myeloid leukemia. *Blood*, **81**, 883-888.

Nucifora G, Larson RA & Rowley JD (1993b) Persistence of the 8;21 translocation in patients with acute myeloid leukemia type M2 in long-term remission. *Blood*, **82**, 712-715.

Nucifora G, Dickstein JI, Torbenson V, Roulston D, Rowley JD & Vardiman JW (1994) Correlation between cell morphology and expression of the AML1/ETO chimeric transcript in patients with acute myeloid leukemia without the t(8;21). *Leukemia*, **8**, 1533-1538.

Nucifora G & Rowley JD (1995) AML1 and the 8;21 and 3;21 translocations in acute and chronic myeloid leukemias. *Blood*, **86**, 1-14.

Ogawa E, Inuzuka M, Maruyama M, Satake M, Naito-Fujimoto M, Ito Y & Shigesada K (1993) Molecular cloning and characterization of PEBP2 beta, the heterodimeric partner of a novel Drosophila runt-related DNA dinding protein PEBP2 alpha. *Virology*, **194**, 314-331.

Okuda T, van Deursen J, Hiebert SW, Grosveld G & Downing JR (1996) AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell*, **84**, 321-330.

Okuda T, Cai Z, Yang S, Lenny S, Lyu CJ, van Deursen JM, Harada H & Downing JR (1998) Expression of a knocked-in AML1-ETO leukemia gene inhibits the establishment of normal definitive hematopoiesis and directly generates dysplastic hematopoietic progenitors. *Blood*, **91**, 3134-3143.

O'Reilly J, Chipper L, Springall F & Herrmann R (2000) A unique structural abnormality of chromosome 16 resulting in a CBF beta-MYH11 fusion transcript in a patient with acute myeloid leukemia, FAB M4. *Cancer Genetics and Cytogenetics*, **121**, 52-55.

Orkin SH (1995) Transcription factors and hematopoietic development. *Journal of Biological Chemistry*, **270**, 4955-4958.

Osato M, Asou N, Abdalla E, Hoshino K, Yamasaki H, Okubo T, Suzushima H, Takatsuki K, Kanno T, Shigesada K & Ito Y (1999) Biallelic and heterozygous point mutations in the runt domain of the AML1/PEBP2αB gene associated with myeloblastic leukemias. *Blood*, **93**, 1817-1824.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB & Owen MJ (1997) Cbfa1. a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*, **89**, 765-771.

Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, Behre G, Hiddeman W & Tenen DG (2001a) Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-α (C/EBPα), in acute myeloid leukemia. *Nature Genetics*, **27**, 263-270.

Pabst T, Mueller BU, Harakawa N, Schoch C, Haferlach T, Behre G, Hiddemann W, Zhang DE & Tenen DG (2001b) AML1-ETO down regulates the granulocyte differentiation factor C/EBPα in t(8;21) myeloid leukemia. *Nature Medicine*, **7**, 444-451.

Pallisgaard N, Hokland P, Riishoj DC, Pedersen B & Jorgensen P (1998) Multiplex reverse transcription-polymerase chain reaction for simultaneous screening of 29 translocations and chromosomal aberrations in acute leukemia. *Blood*, **92**, 574-588.

Paskulin GA, Philips G, Morgan R, Sandberg A, Richkind K, Borovik C, McGavran L, Rabinovich N, Dietz-Band J, Erickson P, Drabkin H & Varella-Garcia M (1998) Pre-clinical evaluation of probes to detect t(8;21) AML minimal residual disease by fluorescence in situ hybridization. *Genes Chromosomes and Cancer*, **21**, 144-151.

Pirc-Danoewinata H, Dauwerse HG, Konig M, Chudoba I, Mitterbauer M, Jaeger U, Breuning MH & Haas OA (2000) CBFB/MYH11 fusion in a patient with AML-M4Eo and cytogenetically normal chromosomes 16. *Genes Chromosomes and Cancer*, **29**, 186-191.

Plantier I, Lai JL, Wattel E, Bauters F & Fenaux P (1994) Inv(16) may be one of the only 'favorable' factors in acute myeloid leukemia: a report on 19 cases with prolonged follow-up. *Leukemia Research*, **18**, 885-888.

Poirel H, Radford-Weiss I, Rack K, Troussard X, Veil A, Valensi F, Picard F, Guesnu M, Leboeuf D & Melle J (1995) Detection of the chromosome 16 CBF beta-MYH11 fusion transcript in myelomonocytic leukemias. *Blood*, **85**, 1313-1322.

Preston DL, Kusumi S, Tomonaga M, Izumi S, Ron E, Kuramoto A, Kamada N, Dohy H & Matsuo T (1994) Cancer incidence in atomic bomb survivors. Part III. Leukemia, lymphoma and multiple myeloma, 1950-1987. *Radiation Research*, **137**, S68-S97.

Preudhomme C, Philippe N, Macintyre E, Henic N, Lai JL, Jouet JP, Cosson A & Fenaux P (1996) Persistence of AML1/ETO fusion mRNA in t(8;21) acute myeloid leukemia (AML) in prolonged remission: is there a consensus? *Leukemia*, **10**, 186-188.

Preudhomme C, Revillion F, Merlat A, Hornez L, Roumier C, Duflos-Grardel N, Jouet JP, Cosson A, Peyrat JP & Fenaux P (1999) Detection of BCR-ABL transcripts in chronic myeloid leukemia (CML) using a 'real time' quantitative RT-PCR assay. *Leukemia*, **13**, 957-964.

Preudhomme C, Warot-Loze D, Roumier C, Grardel-Duflos N, Garand R, Lai JL, Dastugue N, Macintyre E, Denis C, Bauters F, Kerckaert JP, Cosson A & Fenaux P (2000) High incidence of biallelic point mutations in the Runt domain of the AML1/PEBP2αB gene in Mo acute myeloid leukemia and in myeloid malignancies with acquired trisomy 21. *Blood*, **96**, 2862-2869.

Raanani P, Dazzi F, Sohal J, Szydlo RM, van Rhee F, Reiter A, Lin F, Goldman JM & Cross NC (1997) The rate and kinetics of molecular response to donor leucocyte transfusions in chronic myeloid leukaemia patients treated for relapse after allogeneic bone marrow transplantation. *British Journal of Haematology*, **99**, 945-950.

Rabbitts TH (1994) Chromosomal translocations in human cancer. *Nature*, **372**, 143-149.

Reddy KS, Yang X, Mak L, Wang S & Johnston M (2000) A child with ALL and ETV6/AML1 fusion on a chromosome 12 due to an insertion of AML1 and loss of ETV6 from the homolog involved in a t(12;15)(p13;q15). *Genes Chromosomes and Cancer*, **29**, 106-109.

Redner RL, Wang J & Liu JM (1999) Chromatin remodeling and leukemia: new therapeutic paradigms. *Blood*, **94**, 417-428.

Rege K, Swansbury GJ, Atra AA, Horton C, Min T, Dainton MG, Matutes E, Durosinmi M, Treleaven JG, Powles RL & Catovsky D (2000) Disease features in acute myeloid leukemia with t(8;21)(q22;q22). Influence of age, secondary karyotype abnormalities, CD19 status, and extramedullary leukemia on survival. *Leukemia and Lymphoma,* **40**, 67-77.

Rhoades KL, Hetherington CJ, Harakawa N, Yergeau DA, Zhou L, Liu LQ, Little MT, Tenen DG & Zhang DE (2000) Analysis of the role of AML1-ETO in leukemogenesis, using an inducible transgenic mouse model. *Blood*, **96**, 2108-2115.

Richkind K, Hromas R, Lytle C, Crenshaw D, Velasco J, Roherty S, Srinivasiah J & Varella-Garcia M (2000) Identification of two new translocations that disrupt the AML1 gene. *Cancer Genetics and Cytogenetics*, **122**, 141-143.

Ritter M, Thiede C, Schakel U, Schmidt M, Alpen B, Paschenberg U, Mohr B, Ehninger G & Neubauer A (1997) Underestimation of inversion (16) in acute myeloid leukaemia using standard cytogenetics as compared with polymerase chain reaction: results of a prospective investigation. *British Journal of Haematology*, **98**, 969-972.

Roulston D, Espinosa R, Nucifora G, Larson RA, Le Beau MM & Rowley JD (1998) CBFA2 (AML1) translocations with novel partner chromosomes in myeloid leukemias: association with prior therapy. *Blood*, **92**, 2879-2885.

Rowley JD (1973) Identification of a translocation with quinacrine fluorescence in a patient with acute leukemia. *Annals of Genetics*, **16**, 109-112.

Rubnitz JE, Pui CH & Downing JR (1999) The role of TEL fusion genes in pediatric leukemias. *Leukemia*, **13**, 6-23.

Sacchi N, Magnani I, Kearney L, Wijsman J, Hagemeijer A & Darfler M (1995) Interphase cytogenetics of the t(8;21)(q22;q22) associated with acute myelogenous leukemia by two-color fluorescence in situ hybridization. *Cancer Genetics and Cytogenetics*, **79**, 97-103.

Sakata N, Okamura T, Inoue M, Yumura-Yagi K, Hara J, Tawa A, Kodera U, Sako M & Kawa-Ha K (1997) Rapid disappearance of AML1/ETO fusion transripts in patients with t(8;21) acute myeloid leukemia following bone marrow transplantation and chemotherapy. *Leukemia and Lymphoma*, **26**, 141-152.

Santucci MA, Saglio G & Tura S (1996) Pathogenesis and progression of chronic myeloid leukemia. *Haematologica*, **81**, 63-76.

Sasaki K, Yagi H, Bronson RT, Tominaga K, Matsunashi T, Deguchi K, Tani Y, Kishimoto T & Komori T (1996) Absence of fetal liver hematopoiesis in mice deficient in transcriptional coactivator core binding factor beta. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 12359-12363.

Sasaki K, North T, Speck NA & Golub TR (2000) Transcriptional targets of core binding factor fusion proteins. *Blood*, **96**, 301a.

Satake N, Maseki N, Kozu T, Sakashita A, Kobayashi H, Sakurai M, Ohki M & Kaneko Y (1995) Disappearance of AML1-MTG8(ETO) fusion transcript in acute myeloid leukaemia patients with t(8;21) in long-term remission. *British Journal of Haematology*, **91**, 892-898.

Saunders MJ, Tobal K & Liu Yin JA (1994) Detection of t(8;21) by reverse transcriptase polymerase chain reaction in patients in remission of acute myeloid leukaemia. *Leukemia Research*, **18**, 891-895.

Sawyers CL (1999) Chronic myeloid leukemia. *New England Journal of Medicine*, **340**, 1330-1340.

Schiffer CA, Lee EJ, Tomiyasu T, Wiernik PH & Testa JR (1989) Prognostic impact of cytogenetic abnormalities in patients with de novo acute nonlymphocytic leukemia. *Blood*, **73**, 263-270.

Schoch C, Haase D, Haferlach T, Gudat H, Buchner T, Freund M, Link H, Lengfelder E, Wandt H, Sauerland MC, Loffler H & Fonatsch C (1996) Fifty-one patients with acute myeloid leukemia and translocation t(8;21)(q22;q22): an additional deletion in 9q is an adverse prognostic factor. *Leukemia*, **10**, 1288-1295.

Schubauer-Berington MK & Wenzl TB (2001) Leukemia mortality among radiation-exposed workers. *Occupational Health*, **16**, 271-287.

Seymour JF, Juneja SK, Campbell LJ, Ellims PH, Estey EH & Prince HM (1999) Secondary acute myeloid leukemia with inv(16): report of two cases following paclitaxel-containing chemotherapy and review of the role of intensified ara-C therapy. *Leukemia*, **13**, 1735-1740.

Shimizu K, Miyoshi H, Kozu T, Nagata J, Enomoto K, Maseki N, Kaneko Y & Ohki M (1992) Consistent disruption of the AML1 gene occurs within a single intron in the t(8;21) chromosomal translocation. *Cancer Research*, **52**, 6945-6948.

Shurtleff SA, Meyers S, Hiebert SW, Raimondi SC, Head DR, Willman CL, Wolman S, Slovak ML, Carroll AJ & Behm F (1995) Heterogeneity in CBF beta/MYH11 fusion messages encoded by the inv(16)(p13q22) and the t(16;16)(p13;q22) in acute myelogenous leukemia. *Blood*, **85**, 3695-3703.

Siegel R, Gartenhaus R & Kuzel T (2001) HTLV-1 associated leukemia/lymphoma: epidemiology, biology, and treatment. *Cancer Treatment Reviews*, **104**, 75-88.

Sievers EL, Larson RA, Stadtmauer EA, Estey E, Lowenberg B, Dombret H, Karanes C, Theobold M, Bennett JM, Sherman ML, Berger MS, Eten CB, Loken MR, van Dongen JJ, Bernstein ID & Applebaum FR (2001) Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. *Journal of Clinical Oncology*, **19**, 3244-3254.

Slovak ML, Kopecky KJ, Cassileth PA, Harrington DH, Theil KS, Mohamed A, Paietta E, Willman CL, Head DR, Rowe JM, Forman SJ & Appelbaum FR (2000) Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. *Blood*, **96**, 4075-4083.

Song W-J, Sullivan MG, Legare RD, Hutchings S, Tan X, Kufrin D, Ratajczak J, Resende IC, Haworth C, Hock R, Loh M, Felix C, Roy D-C, Busque L, Kurnit D, Willman C, Gerwitz AM, Speck NA, Bushweller JH, Li FP, Gardiner K, Poncz M, Maris JM & Gilliland DG (1999) Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. *Nature Genetics*, **23**, 166-175.

Sonneveld P & List AF (2001) Chemotherapy resistance in acute myeloid leukaemia. *Best Practice and Research Clinical Haematology*, **14**, 211-233.

Sooknanan R, Malek L, Wang XH, Siebert T & Keating A (1993) Detection and direct sequence identification of BCR-ABL mRNA in Ph+ chronic myeloid leukemia. *Experimental Hematology*, **21**, 1719-1724.

Speck NA, Stacy T, Wang Q, North T, Gu T-L, Miller J, Binder M & Marin Padilla M (1999) Core-binding factor: a central player in hematopoiesis and leukemia. *Cancer Research*, **59**, S1789-S1793.

Springall FH, Lukeis RL, Tyrrell V, Joshua DE & Iland HJ (1998) Identification of a novel CBFB-MYH11 fusion transcript in a patient with AML and inversion of chromosome 16. *Leukemia*, **12**, 2034-2035.

Steer EJ, Goldman JM & Cross NC (2001) Mutations of the transcription factor AML1/CBFA2 are uncommon in blastic transformation of chronic myeloid leukaemia. *Leukemia*, **15**, 476-477.

Streubel B, Valent P, Lechner K & Fonatsch C (2001) Amplification of the AML1 (CBFA2) gene on ring chromosomes in a patient with acute myeloid leukemia and a constitutional ring chromosome 21. *Cancer Genetics and Cytogenetics*, **124**, 42-46.

Strick R, Strissel PL, Borgers S, Smith SL & Rowley JD (2000) Dietary bioflavinoids induce cleavage in the MLL gene and may contribute to infant leukemia. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 4790-4795.

Sutherland GR & Richards RI (1995) Simple tandem DNA repeats and human genetic disease. *Proceedings of the National Academy of Sciences of the United States of America*, 92, 3636-3641.

Swansbury GJ, Lawler SD, Alimena G, Arthur D, Berger R, Van den Berghe H, Bloomfield CD, de la Chappelle A, Dewald G & Garson OM (1994) Long-term survival in acute myelogenous leukemia: a second follow-up of the Fourth International Workshop on Chromosomes in Leukemia. *Cancer Genetics and Cytogenetics*, **73**, 1-7.

Swirsky DM, Li YS, Matthews,JG, Flemans RJ, Rees JK & Hayhoe FG (1984) 8;21 translocation in acute granulocytic leukaemia: cytological, cytochemical and clinical features. *British Journal of Haematology*, **56**, 199-213.

Szyrach M, Munchberg FE, Riehle H, Nordheim A, Krauter J, Nagel S, Heil G & Heidenreich O (2001) Cleavage of AML1/MTG8 by asymmetric hammerhead ribozymes. *European Journal of Biochemistry*, **268**, 3550-3557.

Tahirov TH, Inoue-Bungo T, Morii H, Fujikawa A, Sasaki M, Kimura K, Shiina M, Sato K, Kumasaka T, Yamamoto M, Ishii S & Ogata K (2001) Structural analysis of DNA recognition by the AML1/Runx-1 runt domain and its allosteric control by CBFβ. *Cell*, **104**, 755-767.

Tanaka T, Tanaka K, Ogawa S, Kurokawa M, Mitani K, Nishida J, Shibata Y & Hirai H (1995a) An acute myeloid leukemia gene, AML1, regulates hemopoietic myeloid cell differentiation and transcriptional activation antagonistically by two alternatively spliced forms. *EMBO Journal*, **14**, 341-350.

Tanaka T, Mitani K, Kurokawa M, Ogawa S, Tanaka K, Nishida J, Yazaki Y, Shibata Y & Hirai H (1995b) Dual functions of the AML1/EVI-1 chimeric protein in the mechanism of leukemogenesis in t(3;21) leukemias. *Molecular and Cellular Biology*, **15**, 2383-2392.

Tanaka K, Arif M, Asou H, Shimizu K, Ohki M, Kyo T, Dohy H & Kamada N (1999) detection of translocation 8;21 on interphase cells from acute myelocytic leukemia by fluorescence in situ hybridization and its clinical application. *Cancer Genetics and Cytogenetics*, **113**, 29-35.

Tang YY, Crute BE, Kelley JJ, Huang X, Yan J, Shi J, Hartman KL, Laue TM, Speck NA & Bushweller JH (2000) Biophysical characterization of interactions between the core binding factor alpha and beta subunits and DNA. *FEBS Letters*, **470**, 167-172.

Taub JW (2001) Relationship of chromosome 21 and acute leukemia in children with Down syndrome. *Journal of Pediatric Hematology Oncology*, **23**, 175-178.

Taviaux S, Brunel V, Dupont M, Fernandez F, Ferraz C, Carbuccia N, Sainty D, Demaille J, Birg F & Lafage-Pochitaloff M (1999) Simple variant t(8;21) acute myeloid leukemias harbor insertions of the AML1 or ETO genes. *Genes Chromosomes and Cancer*, **24**, 165-171.

Tenen DG, Hromas R, Licht JD & Zhang DE (1997) Transcription factors, normal myeloid development, and leukemia. *Blood*, **90**, 489-519.

Thompson JD, Brodsky I & Yunis JJ (1992) Molecular quantification of residual disease in chronic myelogenous leukemia after bone marrow transplantation. *Blood*, **79**, 1629-1635.

Tighe JE, Daga A & Calabi F (1993) Translocation breakpoints are clustered on both chromosome 8 and chromosome 21 in the t(8;21) of acute myeloid leukemia. *Blood*, **81**, 592-596.

Tighe JE & Calabi F (1994) Alternative, out-of-frame runt/MTG8 transcripts are encoded by the derivative (8) chromosome in the t(8;21) of acute myeloid leukemia M2. *Blood*, **84**, 2115-2121.

Tobal K, Johnson PR, Saunders MJ, Harrison CJ & Liu Yin JA (1995) Detection of CBFB/MYH11 transcripts in patients with inversion and other abnormalities of chromosome 16 at presentation and remission. *British Journal of Haematology*, **91**, 104-108.

Tobal K & Yin JA (1996) Monitoring of minimal residual disease by quantitative reverse transcriptase-polymerase chain reaction for AML1-MTG8 transcripts in AML-M2 with t(8;21). *Blood*, **88**, 3704-3709.

Tobal K & Liu Yin JA (1998) Molecular monitoring of minimal residual disease in acute myeloblastic leukemia with t(8;21) by RT-PCR. *Leukemia and Lymphoma*, **31**, 115-120.

Tobal K, Newton J, Macheta M, Chang J, Morgenstern G, Evans PA, Morgan G, Lucas GS & Liu Yin JA (2000) Molecular quantitation of minimal residual disease in acute myeloid leukemia with t(8;21) can identify patients in durable remission and predict clinical relapse. *Blood*, **95**, 815-819.

Torok-Storb B, Iwata M, Graf L, Gianotti J, Horton H & Byrne MC (1999) Dissecting the marrow microenvironment. *Annals of the New York Academy of Sciences*, **872**, 164-170.

Uckun FM, Herman-Hatten K, Crotty ML, Sensel MG, Sather HN, Tuel-Ahlgren L, Sarquis MB, Bostrom B, Nachman JB, Steinherz PG, Gaynon PS & Heerema N (1998) Clinical significance of MLL-AF4 fusion transcript expression in the absence of a cytogenetically detectable t(4;11)(q21;q23) chromosomal translocation. *Blood*, **92**, 810-821.

van der Reijden BA, Dauwerse JG, Wessels JW, Beverstock GC, Hagemeijer A, van Ommen GJ & Breuning MH (1993) A gene for a myosin peptide is disrupted by the inv(16)(p13q22) in acute nonlymphocytic leukemia M4Eo. *Blood*, **82**, 2948-2952.

van der Reijden BA, Lombardo M, Dauwerse HG, Giles RH, Muhlematter D, Bellomo MJ, Wessels HW, Beverstock GC, van Ommen GJ & Hagemeijer A(1995) RT-PCR diagnosis of patients with acute nonlymphocytic leukemia and inv(16)(p13q22) and identification of new alternative splicing in CBFB-MYH11 transcripts. *Blood*, **86**, 277-282.

van der Reijden BA, Martinet D, Dauwerse JG, Giles RH, Wessels JW, Beverstock GC, Smit B, Muhlematter D, Jotterand Bellomo M, Gabert J, Lafage-Pochitaloff M, Reiffers J, Bilhou-Nabera C, van Ommen GJ, Hagemeijer A & Breuning MH (1996) Simple method for detection of MYH11 DNA rearrangements in patients with inv(16)(p13q22) and acute myeloid leukemia. *Leukemia*, **10**, 1459-1462.

van der Reijden BA, Bloomfield CD, Touw IP & Jansen JH (1997) Acute leukemias with structurally altered core binding factor subunits [t(8;21), inv(16), t(12;21)], 27-28 June 1997, Rotterdam, The Netherlands. *Leukemia*, **11**, 2217-2219.

van Dongen JJ, Macintyre EA, Gabert JA, Delabesse E, Rossi V, Saglio G, Gottardi E, Rambaldi A, Dotti G, Griesinger F, Parreira A, Gameiro P, Diaz MG, Malec M, Langerak, AW, San Miguel JF & Biondi A (1999) Standarized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in leukemia. *Leukemia*, **13**, 1901-1928.

van Oostveen J, Bijl J, Raaphorst F, Walboomers J & Meijer C (1999) The role of homeobox genes in normal hematopoiesis and hematological malignancies. *Leukemia*, **13**, 1675-1690.

van Rhee F, Lin F, Cullis JO, Spencer A, Cross NC, Chase A, Garicochea B, Bungey J, Barrett J & Goldman JM (1994) Relapse of chronic myeloid leukemia after allogeneic bone marrow transplant: the case for giving donor leukocyte transfusions before the onset of hematologic relapse. *Blood*, **83**, 3377-3383.

Veldman T, Vignon C, Schrock E, Rowley JD & Ried T (1997) Hidden chromosomal abnormalities in haematological malignancies detected by multicolour spectral karyotyping. *Nature Genetics*, **15**, 406-419.

Verkerk AJMH, Pieretti M, Sutcliffe JS, Fu Y-H, Kuhl DPA, Pizzuti A, Reiner O, Richards S, Victoria MF, Zhang F, Eussen BE, van Ommen G-JB, Blonden LAJ, Riggins GJ, Chastain JL, Kunst CB, Galjaard H, Caskey CT, Nelson DL, Oostra BA & Warren ST (1991) Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell*, **65**, 905-914.

Viswanatha DS, Chen I, Liu PP, Slovak ML, Rankin C, Head DR & Willman CL (1998) Characterization and use of an antibody detecting the CBFbeta-SMMHC fusion protein in inv(16)/t(16;16)-associated acute myeloid leukemias. *Blood*, **91**, 1882-1890.

Walker H, Smith F & Betts DR (1994) Cytogenetics in acute myeloid leukemia. *Blood Revues*, **8**, 30-36.

Wang SW & Speck NA (1992) Purification of core-binding factor, a protein that binds the conserved core site in murine leukemia virus enhancers. *Molecular and Cellular Biology*, **12**, 89-102.

Wang JYJ (1993) Abl tyrosine kinase in signal transduction and cell-cycle regulation. *Current Opinion in Genetics and Development*, **3**, 35-43.

Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH & Speck NA (1996a) Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 3444-3449.

Wang Q, Stacy T, Miller JD, Lewis AF, Gu TL, Huang X, Bushweller KH, Bories JC, Alt FW, Ryan G, Liu PP, Wynshaw-Boris A, Binder M, Marin-Padilla M, Sharpe AH & Speck NA (1996b) The CBFbeta subunit is essential for CBFalpha2 (AML1) function in vivo. *Cell*, **87**, 697-708.

Wang J, Saunthararajah Y, Redner RL & Liu JM (1999) Inhibitors of histone deacetylase relieve ETO-mediated repression and induce differentiation of AML1-ETO leukemia cells. *Cancer Research*, **59**, 2766-2769.

Warren AJ, Bravo J, Williams RL & Rabbitts TH (2000) Structural basis for the heterodimeric interaction between the acute leukaemia-associated transcription factors AML1 and CBFβ. *EMBO Journal*, **19**, 3004-3015.

Wattjes MP, Krauter J, Nagel S, Heidenreich O, Ganser A & Heil G (2000) Comparison of nested competitive RT-PCR and real-time RT-PCR for the detection and quantification of AML1/MTG8 fusion transcripts in t(8;21) positive acute myelogenous leukemia. *Leukemia*, **14**, 329-335.

Weiden PL, Sullivan KM, Flournoy N, Storb R & Thomas ED (1981) Antileukemic effect of chronic graft-versus-host disease: contribution to improved survival after allogeneic marrow transplantation. *New England Journal of Medicine*, **304**, 1529-1533.

Westendorff JJ, Yamamoto CM, Lenny N, Downing JR, Selsted ME & Hiebert SW (1998) The t(8;21) fusion protein, AML1-ETO, associates with C/EBP-alpha, inhibits C/EBP-alpha-dependent transcription, and blocks granulocyte differentiation. *Molecular and Cellular Biology*, **18**, 322-333.

Wiernik PH, Ashwin M, Hu X-P, Paietta E & Brown K (2001) Anticipation in familial chronic lymphocytic leukaemia. *British Journal of Haematology*, **113**, 407-414.

Willman CL (1998) Molecular genetic features of myelodysplastic syndromes. *Leukemia*, **12**, S2-S6.

Willman CL (2001) Targeted AML therapy: new biological paradigms and therapeutic opportunities. *Leukemia*, **15**, 690-694.

Wolford JK & Prochazka M (1998) Structure and expression of the human MTG8/ETO gene. *Gene*, **212**, 103-109.

Wong KF, Kwong YL & So CC (1998) Translocation (8;20;21)(q22;q13;q22) in acute myeloblastic leukemia with maturation: a variant form of t(8;21). *Cancer Genetics and Cytogenetics*, **101**, 39-41.

Wong KF & Kwong YL (1999) Trisomy 22 in acute myeloid leukemia with monocytic features and cytogenetically cryptic inversion 16. *Cancer Genetics and Cytogenetics*, **109**, 131-133.

Wong O (1995) Risk of acute myeloid leukemia amd multiple myeloma in workers exposed to benzene. *Occupational and Environmental Medicine*, **52**, 380-384.

Wu SQ, Voelkerding KV, Sabatini L, Chen XR, Huang J & Meisner LF (1995) Extensive amplification of bcr/abl fusion genes clustered on three marker chromosomes in human leukemic cell line K-562. *Leukemia*, **9**, 858-862.

Yee K, Anglin P & Keating A (1999) Molecular approaches to the detection and monitoring of chronic myeloid leukemia: theory and practice. *Blood Revues*, **13**, 105-126.

Yeoh AEJ, Williams K, Behm F, Lenny N, Shih L-Y, Harada H, Harada Y, Gilliland G & Downing JR (2000) Somatic mutations of the AML-1 gene are frequent in acute myeloid leukemia with FAB M0 morphology. *Blood*, **96**, 91a.

Yergeau DA, Hetherington CJ, Wang Q, Zhang P, Sharpe AH, Binder M, Marin-Padilla M, Tenen DG, Speck NA & Zhang DE (1997) Embryonic lethality and impairment of haematopoiesis in mice heterozygous for an AML1-ETO fusion gene. *Nature Genetics*, **15**, 303-306.

Yuan Y, Zhou L, Miyamoto T, Iwasaki H, Harakawa N, Hetherington CJ, Burel SA, Lagasse E, Weissman IL, Akashi K & Zhang D-E (2001) AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 10398-10403.

Zent C, Kim N, Hiebert S, Zhang DE, Tenen DG, Rowley JD & Nucifora G (1996) Rearrangement of the AML1/CBFA2 gene in myeloid leukemia with the 3;21 translocation: expression of co-existing multiple chimeric genes with similar functions as transcriptional repressors, but with opposite tumorigenic properties. *Current Topics in Microbiology and Immunology*, **211**, 243-252.

Zhang DE, Hetherington CJ, Meyers S, Rhoades KL, Larson CJ, Chen HM, Hiebert SW & Tenen DG (1996) CCAAT enhancer-binding protein (C/EBP) and AML1 (CBFα2) synergistically activate the macrophage colony-stimulating factor receptor promoter. *Molecular and Cellular Biology*, **16**, 1231-1240.

Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ & Tenen DG (1997) Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 569-574.

Zhang T, Hillion J, Tong JH, Cao Q, Chen SJ, Berger R & Chen Z (1994) AML-1 gene rearrangement and AML-1-ETO gene expression as molecular markers of acute myeloblastic leukemia with t(8;21). *Leukemia*, **8**, 729-734.

Zhang YW, Bae SC, Huang G, Fu YX, Lu J, Ahn MY, Kanno Y, Kanno T & Ito Y (1997) A novel transcript encoding an N-terminally truncated AML1/PEBP2α protein interferes with transactivation and blocks granulocytic differentiation of 32Dcl3 myeloid cells. *Molecular and Cellular Biology*, **17**, 4133-4145.

Zwick E, Bange J & Ullrich A (2001) Receptor tyrosine kinase signalling as a target for cancer intervention strategies. *Endocrine Related Cancer*, **8**, 161-173.

Publications arising from work in this thesis

Langabeer SE, Walker H, Wheatley K, Burnett AK, Goldstone AH & Linch DC (1996) Frequency of *CBFB-MYH11* fusion transcripts in acute myelomonocytic leukaemia (AML M4). *British Journal of Haematology*, **93**, Supplement 1, 235 (abstract).

Langabeer SE, Walker H, Gale RE, Wheatley K, Burnett AK, Goldstone AH & Linch DC (1997) Frequency of *CBFB-MYH11* fusion transcripts in patients entered into the UK MRC AML trials. *British Journal of Haematology*, **96**, 736-739.

Langabeer SE, Walker H, Burnett AK, Wheatley K, Goldstone AH & Linch DC (1997) Frequency of *AML1-ETO* fusion transcripts in acute myeloid leukaemia. *British Journal of Haematology*, **97**, Supplement 1, 191 (abstract).

Langabeer SE, Walker H, Rogers JR, Burnett AK, Goldstone AH & Linch DC (1997) Incidence of *AML1-ETO* and *CBFB-MYH11* fusion genes in patients entered into the UK MRC acute myeloid leukaemia trials. *Leukemia*, **11**, 2228, 24 (abstract).

Langabeer SE, Walker H, Rogers JR, Burnett AK, Wheatley K, Swirsky D, Goldstone AH & Linch DC (1997) Incidence of *AML1-ETO* fusion transcripts in patients entered into the MRC AML trials. *British Journal of Haematology*, **99**, 925-928.

Burnett AK, Wheatley K, Langabeer S, Walker H, Linch D, Goldstone AH, Hann I & Stevens RF (1997) Outcome of acute myeloid leukaemias affecting core binding factor subunits: the UK MRC experience. *Blood*, **90**, Supplement 1, 1737 (abstract).

Langabeer SE, Grimwade D, Walker H, Rogers JR, Burnett AK, Goldstone AH & Linch DC (1998) A study to determine whether trisomy 8, deleted 9q and trisomy 22 are markers of cryptic rearrangements of *PML-RAR_*, *AML1-ETO* and *CBFB-MYH11* respectively in acute myeloid leukaemia. *British Journal of Haematology*, **101**, 338-340.

Langabeer SE, Gale RE, Mackinnon S & Linch DC (1999) Transcription mediated amplification and hybridisation protection assay to determine *BCR-ABL* levels in patients with chronic myeloid leukaemia. *Blood*, **94**, Supplement 1, 1267 (abstract).

Langabeer SE, Gale RE, Walker H & Linch DC (2000) Reported cryptic rearrangements of *CBFB-MYH11* in acute myeloid leukaemia are not due to false priming of contaminating DNA. *Leukemia*, **14**, 944-945 (letter).

Langabeer SE, Gale RE, Rollinson SJ, Morgan GJ & Linch DC (2000) Mutations of *AML1* gene in acute myeloid leukaemia of FAB types M0 and M7. *Blood*, **96**, 698 (abstract).

Langabeer SE, Gale RE, Harvey RC, Cook RW, Mackinnon S & Linch DC (2002) Transcription mediated amplification and hybridisation protection assay to determine *BCR-ABL* levels in patients with chronic myeloid leukaemia. *Leukemia*, **16**, 393-399.

Langabeer SE, Gale RE, Rollinson SJ, Morgan GJ & Linch DC (2002) Mutations of the *AML1* gene in acute myeloid leukaemia of FAB types M0 and M7. *Genes Chromosomes and Cancer*, **34**, 24-32.

Langabeer SE, Gale RE & Linch DC (2002) Quantification of *AML1-ETO* transcripts in patients with acute myeloid leukaemia by transcription-mediated amplification and hybridisation protection assay. *British Journal of Haematology*, **117**, Supplement 1, 55 (abstract).