MOLECULAR AND GENE EXPRESSION STUDIES OF THE GENES INVOLVED IN THE BREAKPOINTS OF THE INV(16) LEUKAEMIAS

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A thesis submitted for the degree of Doctor of Philosophy of the University of Adelaide

August, 1996

ABSTRACT

Molecular and gene expression studies of the genes involved in the breakpoints of the inv(16) leukaemias.

B.J. Kuss

Myelomonocytic leukaemia (M4) is one of the commonest subclasses of the acute myeloid leukaemias (AML) representing almost 30% of the total. The M4 eosinophilic variant is characterized by abnormalities of the long arm of chromosome 16. In particular a pericentric inversion of 16: inv(16)(p13q22) and less frequently, rearranged chromosome 16 homologues t(16;16)(p13;q22). In the initial stages of this thesis, strategies for positional cloning of the inv(16) long arm breakpoint were employed. A contiguous piece of cloned DNA, approximately 600kb in length, was identified by the use of two markers (ACH207 and LE12), initially thought to flank the long arm breakpoint of the chromosome 16 inversion. However, both were subsequently demonstrated to be proximal to the breakpoint. Several CpG islands were identified in the cloned DNA, indicating the region proximal to the long arm breakpoint of the inv(16) is likely to be gene rich. This approach was discontinued when the inv(16) breakpoint was cloned by Liu *et al* 1993.

Acute myeloid leukaemia (AML) associated with the inversion chromosome 16 inv(16)(p13q22) has a favourable prognosis and is known to be chemosensitive. Subsequent work from this thesis demonstrated that in a subset of inv(16) AML patients, inversion also resulted in loss of the gene for the multidrug resistance protein (*MRP*) at the short arm breakpoint. The *MRP* gene was mapped to 16p13.13, centromeric to the primary short arm breakpoint, separated from *MYH11* by a distance of approximately 150kb. Deletion of the *MRP* gene was demonstrated by fluorescence *in situ* hybridisation (FISH) to chromosome metaphases, gene dosage studies and by loss of heterozygosity of a flanking microsatellite

marker (*D16S405*). A cohort of twenty two patients with inv(16) leukaemia were analysed for deletion of the *MRP* gene and gene deletion was detected in seven of these patients. By analysis of clinical data it was demonstrated that deletion of the *MRP* gene was significantly associated with a longer time from diagnosis until failure, as determined by death or relapse from complete remission ($p=0.007$). The results of this work suggested that *MRP* deletion as detected by molecular analysis, may play a key role in determining the ultimate outcome in inv(16) AML patients.

The final studies of this thesis primarily concerned the semi-quantitation of *MRP* expression, using reverse transcription polymerase chain reaction in a range of normal and leukaemic peripheral blood and bone marrow cells. RNA expression, designated in arbitrary units with CD34-positive cell expression taken as 1, was found to vary from 0.02 to 10 within the above populations. *MRP* gene deletion status of the inv(16) leukaemias was assessed by interphase FISH and Southern analysis. Those leukaemias shown to have a deletion of one *MRP* allele, expressed normal or low levels of *MRP* mRNA, when compared with a normal CD34 positive population. Variable expression was found in those inv(16) leukaemias where no deletion of an MRP allele was detected in association with the inversion chromosome 16. The results of this study suggest that deletion of an *MRP* allele may result in reduced expression of *MRP* mRNA however the presence of the inversion breakpoint alone is associated with variable expression of *MRP*.

DECLARATION

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

I give my consent to this thesis being made available for loan and photocopying if accepted for the award of the degree.

Bryone Jean Kuss

15.8.96

ACKNOWLEDGEMENTS

The work presented in this thesis was carried out in the Department of Cytogenetics and Molecular Genetics, Women's and Children's Hospital, North Adelaide, South Australia. Funding for the project was provided by the Women's and Children's Hospital, Clinical Research Fellowship(1992-1993), and the National Health and Medical Research Council of Australia (1994-1996).

My supervisors for this project were Associate Professor David Callen and Professor Grant Sutherland. I am indebted to them for giving me the opportunity to work in their department and for their support and guidance throughout my candidature. In particular, David provided frequent opportunities for discussion and allowed a degree of research freedom which was appreciated. In addition, I would like to thank Grant for his "open door policy" with regard to his office and his ability to drop what he is doing, listen thoughtfully to what is said and then to offer meaningful advice. This was always greatly appreciated.

I would like to thank the many departmental members who have provided a friendly and productive working environment over the four and one half years of my candidature and to thank all those in the department who assisted me during the course of this project. In particular Dr. Rob Richards for discussions on various molecular approaches possible for difficult sections of the project; to Melanie Pritchard, Michael Lynch and Eric Kremer for technical advice; Naras Lapsys for the information provided regarding early pulsed field electrophoresis work involving the LE12 and ACH207 probes; Julie Nancarrow for part of the sequencing of the initial long arm breakpoint probes, for the CCG repeat analysis of the *MRP* gene and for assistance with the mapping of the *MRP* gene; Scott Whitmore for technical assistance; Helen Eyre for all of the FISH work included in this thesis and for her technical excellence; Joanna Crawford for the many laboratory favours during the writing up

of the thesis and to Sinoula Apostalou, my friend and colleague for all of her help on technical, theoretical and philosophical aspects of the project. I would like to thank the patients who made available their bone marrow for research purposes and the colleagues in the department who donated their blood for peripheral blood mononuclear cell preparations. In addition I would like to thank Heather and Jenny for their secretarial and computer assistance.

My thanks also extend to the various collaborators which have made this project viable: Professor Roger Deeley and Dr Susan Cole for providing discussion on aspects of multidrug resistance, for the MRP probes and for the use of their laboratories during my stay in Canada: Dr Cheryl Willman for providing the leukaemic samples from the SWOG repository and for welcoming me into her laboratory; Dr Kenneth Kopecky for his excellent statistical advice; Dr Bert van der Reiden and Professor Martijn Breuning for their kind provision of the MYH11 positive cosmids and YACS, the cDNA probe LISP2 and the primers for aberrant transcript detection in inv(16) leukaemias and Dr Ross Davey and Geraldine O'Neil for their provision of the *ARA* probe and open discussions about their recent discovery of the *ARA* gene.

Finally, I would like to thank Jeffrey and our family without whose dedicated and unwavering support this thesis would never have been completed. Thank you for always being there. All my love to all of you.

This thesis is dedicated to Jeffrey, Siobhan, Jean and John.

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ABBREVIATIONS

^{32}P	^{32}-Phosphorus
AML	acute myeloid leukaemia
APL	acute promyelocytic leukaemia
ARA	anthracycline resistance associated protein
AraC	arabinosyl cytosine
ATP	adenosine 5′-triphosphate
BM	bone marrow
BSA	bovine serum albumin
CBF	core binding factor
CD	cluster of differentiation
CHO	chinese hamster ovary
CI	confidence interval
CSF	colony stimulating factor
del	deletion
DEPC	diethylpyrocarbonate
DNR	daunorubicin
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FAB	French American British
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FISH	fluorescence *in situ* hybridisation
GM-CSF	granulocyte-macrophage colony-stimulating factor
g	gravity
HLA-DR	human leukocyte antigen D related

HBSS	Hanks balanced salt solution
Hu	human
inv	inversion
kb	kilobases
kDa	kilodalton
L	litres
LRP	lung resistance-associated protein
Mb	megabase
MDR	multidrug resistance
mM	milli molar
mYAC	mega yeast artificial chromosome
MYH11	myosin heavy chain gene 11
mL	milli litres
MHC	major histocompatibility complex
MRP	multidrug resistance protein
NEC	non erythroid cells
nt	nucleotide
PBMNC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PKC	protien kinase C
R	receptor
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SWOG	South Western Oncology Group
t	translocation
TFRR	transferrin receptor

vs	versus
WCC	white cell count
YAC	yeast artificial chromosome

PUBLICATIONS ARISING

Identification of inv(16) coexisting with an isochromosome 22q by in situ hybridisation in the case of childhood ANLL M4E. Gadd S G, Callen D, Kuss B J. Downing JR, Behm F, Head D, Ribeiro RC, Raimondi SC. Leukemia, 7(10): 1658-1662, 1993.

Deletion of gene for multidrug resistance in acute myeloid leukaemia with inversion in chromosome 16: prognostic implications. Kuss B J, Deeley RG, Cole SPC, Willman C, Kopecky KJ, Wolman SR, Eyre HJ Lane SA, Nancarrow JK, Whitmore SA, Callen DF. Lancet, 343: 1531-1534, 1994.

The biological significance of the multidrug resistance gene MRP in inversion 16 leukaemias. Kuss BJ, Deeley RG, Cole SPC, Willman C, Kopecky KJ, Wolman SR, Eyre HJ, Callen DF. Leukaemia and Lymphoma, 20: 357-364, 1996.

ARA, a new and novel multidrug resistance gene, is also deleted in the inv(16) leukaemias. Kuss BJ, O'Neil G, Eyre H, Longhurst TJ, Callen DF, Davey RA. Submission to Blood August 1996.

ABSTRACTS

Human Genomic Conference - Newcastle 1992 (Poster)

Towards cloning the breakpoint of inversion chromosome 16 seen in M4Eo

American Genetics Society Conference - 1992 (Poster)

Towards cloning the breakpoint of inversion chromosome 16 seen in M4Eo

American Association For Cancer Research - San Francisco 1994

Multi-drug resistance symposium (Presented by Dr Susan Cole)

XVI International Cancer Congress - New Delhi 1994

Multidrug resistance symposium (Presented by Dr Susan Cole)

INTRODUCTION

Chapter 1

1.1 Biological aspects of leukaemia

1.1.1 Stem cell theory

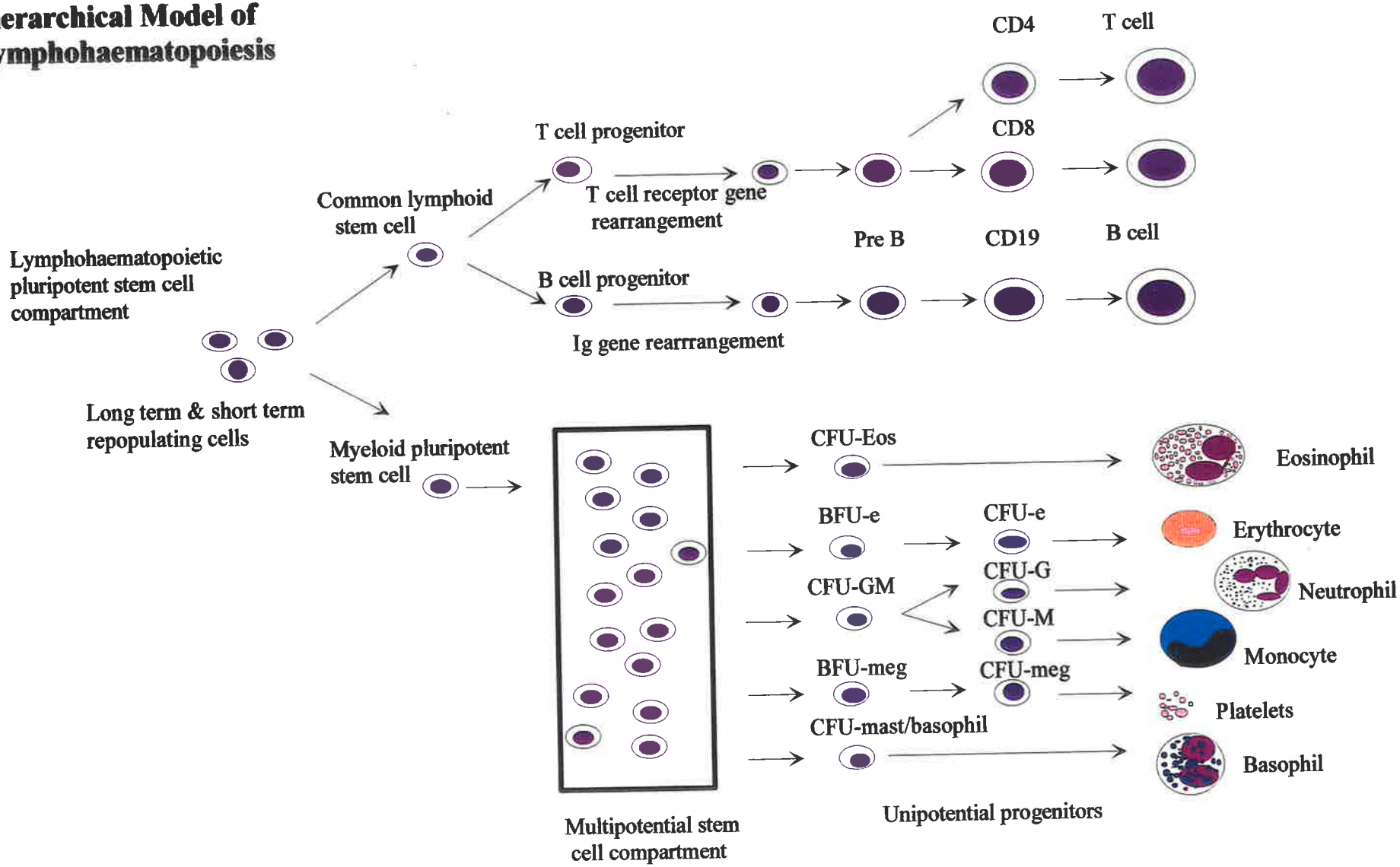
Normal lymphohaematopoiesis is polyclonal and arises from a multipotential stem cell compartment which has both replicative and generative capabilities. Daughter cells are produced which may differentiate into mature cells of a number of different lineages including granulocytes, macrophages, erythroid cells, megakaryocytes or lymphocytes (figure1.1). In the case of fetal haematopoiesis at least, stromal cells may also result. The multipotent stem cell is not morphologically distinct, but has been characterised in a number of ways including biological assays and immunophenotyping. Biological assays include the Colony Forming Unit-Spleen (CFU-S) assay , in which cells of bone marrow (or blood or spleen) are prepared as a single cell suspension and a minimum of 40,000 cells are injected into the tail vein of lethally irradiated mice (Till 1961). At various intervals the mice are killed and their spleens harvested and fixed. Nodules within the spleen are counted and analysed, representing colonies arising from single cells.

Other assays demonstrating stem cell haematopoiesis are cultures in soft agar or methyl cellulose delineating the colony forming unit-blast (CFU-B) and the high proliferative potential - colony forming cell (HPP-CFC) (Bradley 1980). These stem cells require two or more cytokines to form colonies. Another primitive cell, the long term culture initiating cell (LTC-IC) is capable of repopulating the marrow of lethally irradiated mice and is defined by its growth on irradiated marrow stromal cells in a long term culture system (Dexter 1974, Sutherland 1990). Primitive cells have also been characterized in terms of the surface

Figure 1.1 Hierarchical model for lymphohaematopoiesis

A primitive lymphohaematopoietic stem cell is capable of producing stem cells for lymphopoiesis or haematopoiesis. The next generation cells give rise to a series of progressively more differentiated progenitor cells until finally giving rise to a generation of functional end cells for each lineage. The existence of a stem cell common to B and T cells (and presumably natural killer cells) and never been established experimentally. Short term repopulating cells refer to cells that give immediate and short-lived repopulation of the bone marrow when transplanted into experimental animals after irradiation or myelo-ablative chemotherapy. Long term repopulating cells give rise to permanent repopulation of the bone marrow. The multipotential stem cell compartment gives rise to those differentiated cells and functional end cells as listed. CFU - colony forming unit: e = erythroid, G = granulocyte, M = macrophage, meg = megakaryocyte. BFU - burst-forming unit. (After *Quesenberry, PJ.* **Williams Hematology 1995**)

Hierarchical Model of Lymphohaematopoiesis



membrane antigens, cell cycle and metabolic status, giving rise to a phenotype which signifies a primitive generative cell by less lengthy assays than mentioned above. For example, the immunophenotype $CD34^{+}CD38^{-}$ lin^{-} DR^{-} present in human fetal marrow can give rise to stromal cells as well as lymphoid and haematopoietic cells and may give rise to a LTC-IC type (Huang 1992).

Stem cell disorders form a group of blood disorders in which normal haematopoiesis is disrupted. The type of disorder for example acute leukaemia or aplastic anaemia, depends upon the nature of the genetic lesion and the differentiation status of the cell in which the lesion occurs. Acute myeloid leukaemia represents a disorder of a single stem cell and is characterised by the accumulation of abnormal leukaemic blasts present predominantly in the bone marrow, and the impaired production of normal blood cells. It was first demonstrated to be a clonal disorder using G6PD isoenzyme studies (Fialkow 1989). Subsequently other methodologies have been utilised to demonstrate its clonal origin including cytogenetic analysis, which reveal abnormalities such as monosomy 7 and trisomy 8 (Rowley 1990). Molecular biological techniques which demonstrate molecular details of translocations and gene rearrangements such as the immunoglobulin and T cell receptor genes are one step further in the identification of clonal expansion.

1.1.2 The Genetic Basis of Cancer

Cancer may be defined as a progressive series of genetic events that occur in a single cell which eventually result in altered cell kinetics and cell turnover, predisposing to the increased longevity of a clone of cells. In the case of leukaemia, it results in the predominance of the

leukaemic blast cells within the bone marrow to the exclusion of normal non-clonal haematopoiesis. The specific gene types which are affected by these genetic events are labelled oncogenes or tumour suppressor genes. Cellular genes activated by dominant mutations are called proto-oncogenes, reaching full oncogenic potential by a variety of genetic events such as mutations, small insertions or deletions but most commonly by juxtaposition to other genes through translocations or inversions. For example *MYC* (transcription factor) and *ABL* (Abelson proto-oncogene - a tyrosine protein kinase) are involved in the t(9;22)(q34;q11) seen in chronic myeloid leukaemia, and result in the formation of aberrant gene transcripts (Adams 1992). Tumour suppressor genes, facilitate the development of cancer through a recessive mechanism, involving loss of one allele through deletion or mutation followed by a mutation and loss of function of the second allele. The best example of this in leukaemia is the p53 gene, a transcription factor located at 17p13 (Hu 1992). This mechanism has been most widely identified in solid tumours, however it may be that monosomy 7 and the 5q- chromosomal abnormalities result in clonal haematopoiesis by such a mechanism.

Other secondary genetic events occur which may change the characteristics of the malignancy and result in tumour progression. This may be due to the development of drug resistance; a more rapid cell cycle time; or the ability of the tumour to metastasise. For example, the cells of chronic myeloid leukaemia may initially have as their only chromosomal abnormality the t(9;22), then subsequently develop an isochromosome 17q clonal abnormality heralding the onset of blastic transformation (O'Malley 1985). Some genetic events may be easily identified by microscopic alterations of chromosomes. However, it is likely that many more

genetic events which affect the biology of the malignant disease, are occurring at the sub-microscopic level.

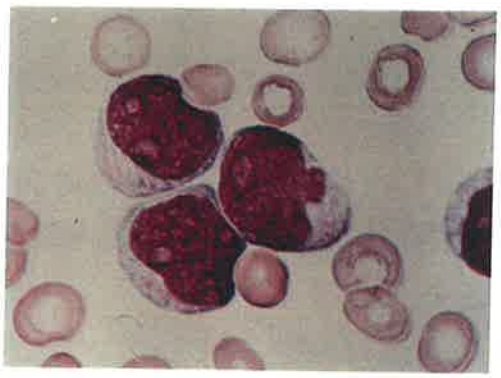
1.2 Cytogenetic analysis of leukaemia and implications for survival

The current classification system for acute myeloid leukaemia, the (FAB) French American British classification system (figure 1.2), is based on light microscopy morphological characteristics only, using Romanowski and cytochemical stains. It divides acute leukaemia into myeloid (AML) and lymphoid (ALL) types with subcategories for each group. This has the limitation of failing to differentiate morphologically similar but aetiologically diverse subtypes of leukaemia, and offers little predictive capability in terms of disease response to chemotherapy and survival of the patient. Cytogenetic analysis has proved to be more informative in terms of subclassification of the leukaemias and the relationship of this to outcome (Walker 1994, Pederson-Bjergaard 1990). With initial chromosomal banding studies (Quinacrine-banding) in the late 1960's and early 1970's, structural organisation of the chromosomes became possible allowing clonal chromosomal rearrangements to be detected in about 50% of cases of AML. Currently, using improved banding techniques (G-banding: Geimsa stained and most commonly, trypsonised chromosomes) and short term unstimulated culture techniques, that figure has risen to 80% in most laboratories (Williams 1995). Chromosomal abnormalities in malignant cells are now described according to the International System for Human Cytogenetic Nomenclature (Mitelman 1995). Although monosomy 7 and trisomy 8 are the most frequently encountered clonal abnormalities, other recurring abnormalities are noted to be associated with specific FAB subclasses and are listed

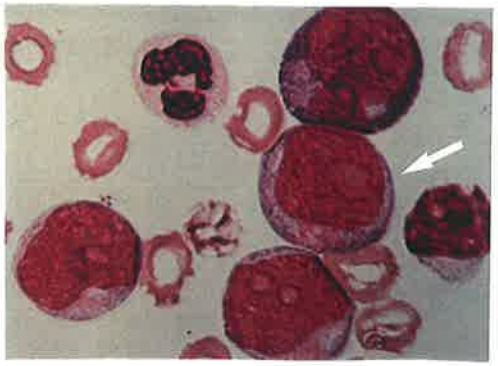
Figure 1.2 (a & b)FAB classification system for Acute Myeloid Leukaemia

The French American British classification system for light microscope diagnosis of AML is listed briefly in the two page figure. Photomicrographs are shown. M1 is characterised by bone marrow aspirates showing blasts with large, often irregular nuclei with one or more nucleoli and varying amounts of eccentrically placed cytoplasm. M2 leukaemic blasts are similar to those of M1, but type 2 blasts containing few granules but no golgi clearing (arrowed) and promyelocytes with azurophilic granules can be seen. In M3, numerous Auer rods may be present in single cells known as faggot cells (arrowed). M4 blast cells have a mixture of monocytic and myelocytic features and in M5 AML, the blasts have monocytoid features with pale blue cytoplasm and non-specific esterase, cytochemical staining. M6 (erythroblastic leukaemia) and M7 (megakaryoblastic leukaemia) are rare but have very typical features as shown. Blasts of an M7 leukaemia may have cytoplasmic "blebs". NEC = non erythroid cells. BM = bone marrow. PB = peripheral blood. (After *Hoffbrand, AV. and Pettit, JE.* **Clinical Haematology Atlas 1988)**

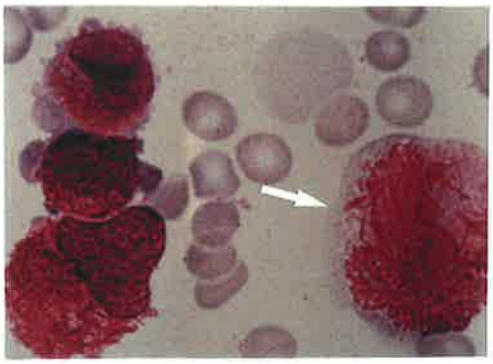
M1 (AML without maturation)
blasts > 90% of NEC; >3% blasts positive for peroxidase or sudan black B; monocytic and/or granulocytic component <10% of NEC each.



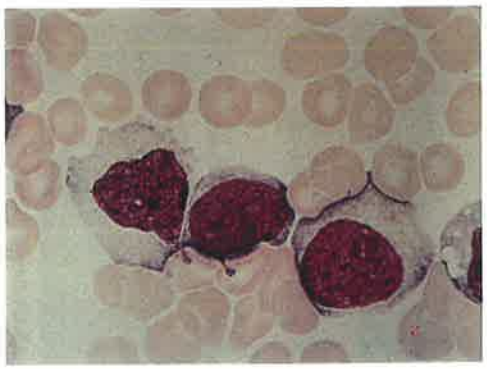
M2 (AML with granulocytic maturation)
blasts 30-89% of NEC; monocytic component <20% of NEC.



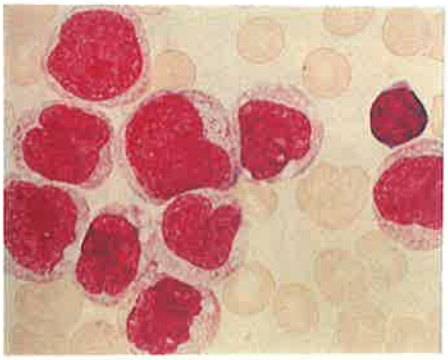
M3 & variant (Acute Promyelocytic Leukaemia)
Promyelocytes with prominent granules >50%; Auer rods and occasional faggot cells. Variant is microgranular with abnormally lobed nuclei.



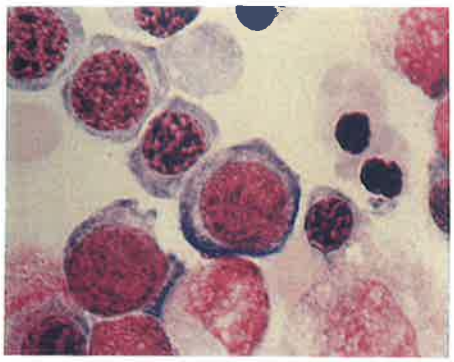
M4 (Acute Myelomonocytic Leukaemia)
blasts 30-89% of NEC; granulocytic component >20% of NEC including myeloblasts and BM monocytic component >20% with PB monocyte count >$5x10^9$/L or confirmation of monocytic component by cytochemical or biochemical means.



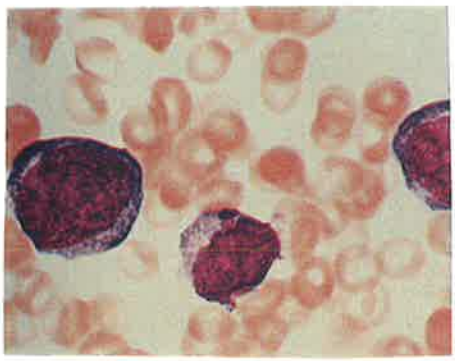
M5a & b (Acute Monocytic Leukaemia)
Monocytic component >80% of NEC. Monoblasts >80% of monocytic component. M5a denotes monblasts are large with lacy chromatin and abundant cytoplasm ie. primitive appearance. M5b denotes evidence of monocytic differentiation present.



M6 (Erythroleukaemia)
Erythroblasts > 50%, blasts > 30% of NEC.



M7 (Megakaryoblastic Leukaemia)
Blasts demonstrated to be megakaryoblasts eg. by ultrastructural cytochemistry or by immunological means (presence of platelet antigens).



in table 1.1. These abnormalities assist in the further sub-classification of AML with a greater emphasis on aetiology and better association with outcome than the FAB classification alone.

Survival figures for acute leukaemia have improved in comparison to historical controls partly due to the ability to deliver greater chemotherapeutic dose intensity (Gale 1990, Watilin 1991). However, relapse remains a dismal indicator of the prospects for the patient and is likely to be attributable, at least in part, to the expression of multidrug resistance (MDR). At present, the best prognostic indicators available for acute myeloid leukaemias are remission status after course one of chemotherapy, abnormalities of karyotype and age of onset. Cytogenetically, good prognosis has been associated with t(15;17)(q22;q12), t(8;21)(q22;q22) and inv(16)(p13.1q22.1) (LeBeau 1983, Raimondi 1989, Larson 1986). The poor prognostic group includes 5q-, 7q-, monosomies of chromosomes 5 or 7, and complex karyotypes. The 5 year survival figures for good and poor prognostic groups are of the order of 71% and 20%, respectively. The presence of a favourable cytogenetic profile is of greater significance than the presence of residual disease after the completion of the first course of chemotherapy (Rassam 1993).

1.3 Historical background of M4Eo as a histopathological entity

Myelomonocytic leukaemia (M4) is one of the commonest subclasses of the acute myeloid leukaemias (AML) representing almost 30% of the total. It is characterised by a bone marrow white cell differential containing a minimum of 20% myeloblasts plus promyelocytes and a reciprocal number of monoblasts and monocytoid cells. Other features are listed in figure 1.3.

Table1.1 Chromosomal abnormalities in Acute Myeloid Leukaemia

Disease	Chromosome abnormality	Frequency of chromosomal abnormality in AML (%)
AML-M2	t(8;21)(q22;q22)	12.8
APL-M3	t(15;17)(q22;q12)	12
AMMoL - M4Eo	inv(16)(p13q22) or t(16;16)(p13;q22)	8.7
AMMoL - M4 / **AMoL - M5**	t(9;11)(p22;q23) t(10;11)(p11-p15;q23) t(11;17)(q23;q25) t11;19)(q23;p13) other t(11q23) del(11)(q23)	7.5
AML	+8 +21 -7 or del(7q) -5 or del(5q) -Y t(6;9)(p23;q34) t(3;3)(q21;q26) or inv(3)(q21q26) del(20q) t(12p) or del(12p)	15.2 5 15.7 6 7.5 1.0 1.0 1.5 3.4
Therapy-related AML	-7 or del(7q) and/or -5 or del(5q) t(11q23) der(1)t(1;7)(q10;p10)	75 3 2

Chromosomal abnormalities are described as seen in acute myeloid leukaemia (AML), classified according to FAB classification criteria. The karyotypes are written as per an International System for human Cytogenetic Nomenclature (ISCN). AML-M2 (AML with granulocytic maturation). APL-M3 (acute promyelocytic leukaemia). AMMoL-M4 (acute myelomonocytic leukaemia). M4Eo (M4 variant: with abnormal bone marrow eosinophilia). AMoL (acute monocytic leukaemia). Therapy related AML (secondary to cytotoxic or radiation therapy) is commonly associated with abnormalities of chromosomes 5 and 7.

Figure 1.3 Myelomonocytic leukaemia with abnormal eosinophils (M4Eo)

The clinical, cytologic and karyotypic features of M4Eo are presented. The photomicrograph shows the abnormal eosinophil with basophilic granulation (arrowed) and blast cells immediately below and to the upper left. The karyotype of this metaphase spread derived from a patient with M4Eo leukaemia is 47,XY,+8,inv(16)(p13q22). The normal chromosome 16 is arrowed and labelled "16" and the inv(16) is arrowed and labelled "i". The three chromosomes 8 are shown.

Clinical Features

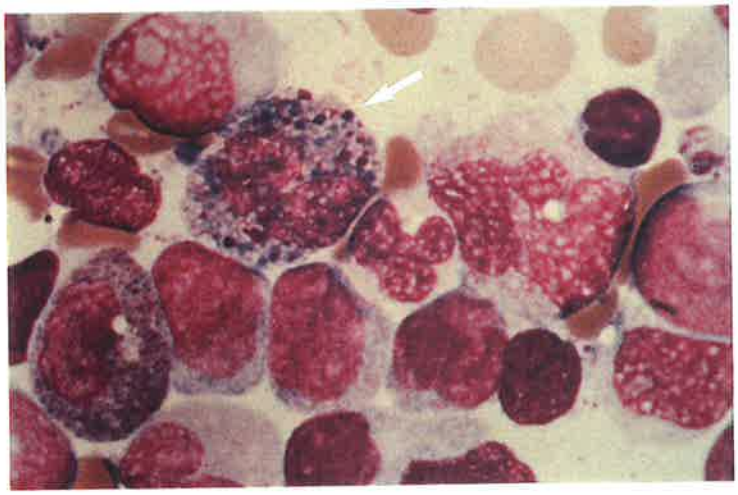
1. Most common in adults 5-10% of AML however significant incidence in children

2. Frequent extramedullary disease & risk of CNS relapse

3. Slightly elevated serum and urine muramidase levels

Cytologic Features

1. Both myeloblastic and monoblastic leukaemic cells in blood and bone marrow



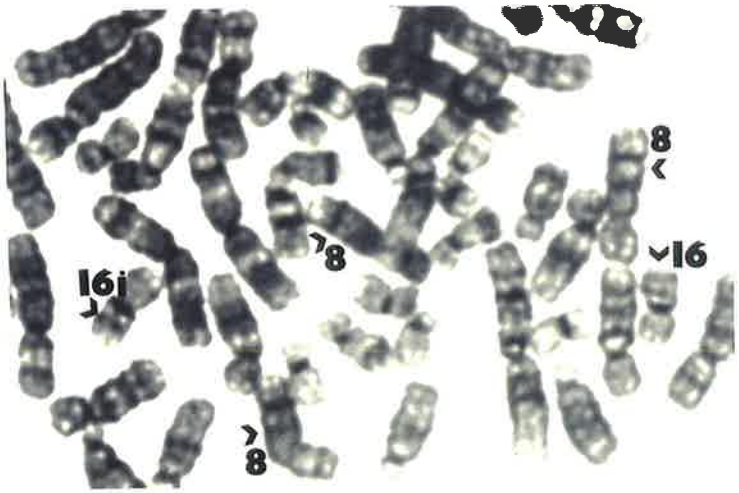
2. Peroxidase, Sudan Black, Chloroacetate and non-specific esterase positive cells

3. Bone marrow eosinophilia with abnormal granules

4. Specific immuno- phenotype: including CD2 differentiating M4Eo/inv(16) from M4 without inv(16)

Karyotypic Features

1. Association with the
inv(16)(p13.1,q22.1)
t(16;16)(p13.1;q22.1)
del(16)(q22.1)



2. 16q abnormalities detected in 12% of paediatric AML

3. Numerical abnormalities also found:+8,+22,+9,-2,-20

This subclass is variably associated with a number of non-random cytogenetic abnormalities. However, the M4 eosinophilic variant (M4Eo) with its abnormal bone marrow eosinophilia, is characterized by abnormalities of the long arm of chromosome 16. These are a pericentric inversion of 16, inv(16)(p13.13q22.1), rearranged chromosome 16 homologues, t(16;16)(p13;q22) (LeBeau 1983) and a deletion of the long arm of chromosome 16, del(16q22) (Arthur 1982). Le Beau first published data concerning this chromosomal rearrangement and acute myelomonocytic leukaemia in 1983, describing it as a clinicopathological entity (figure 1.3). Arthur and Bloomfield had previously reported an association between the deletion of the long arm of chromosome 16 (del(16q22)) and a morphologically identical leukaemia in 1982. Acute leukaemias associated with the inv(16)(p13q22.1) and t(16;16)(p13;q22.1), are known to be part of a favourable prognostic group in AML (Larson 1986) (figure 1.4). These are in general chemosensitive, with a longer relapse free survival after induction chemotherapy compared with other acute myeloid leukaemias, although there is some question about their propensity to relapse in the central nervous system (Ohyashiki 1988). The leukaemias described by their association with the del(16q22), are a separate prognostic group which respond less favourably to chemotherapy (Larson 1986, P. Marlton - personal communication concerning the MD Anderson Cancer Center, Houston, experience). A further understanding of the molecular basis of these diseases may clarify the observed differences in their biology and prognoses.

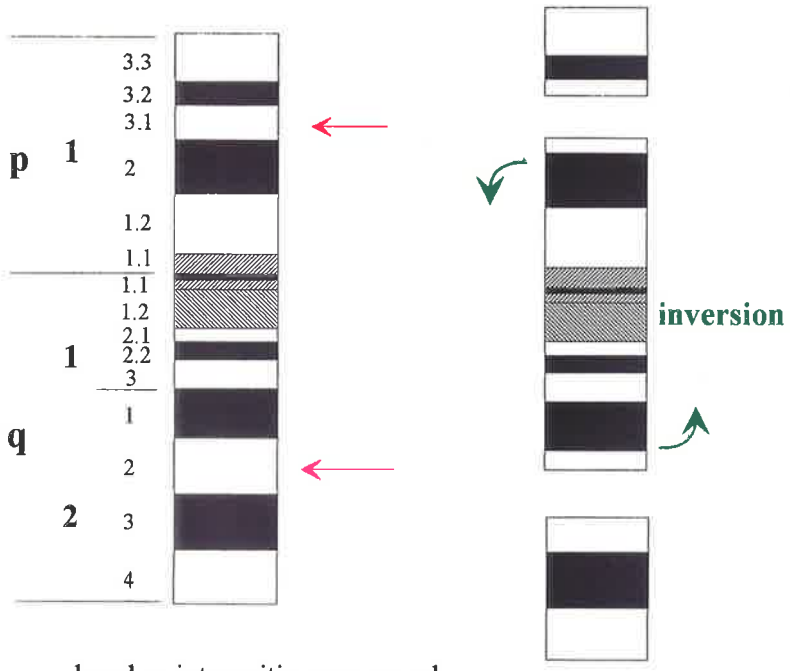
Initially the long arm breakpoint of the inversion chromosome 16 rearrangement was believed to interrupt the metallothionein gene complex, a family of proteins of low relative molecular mass which bind heavy metals (LeBeau 1985). These were postulated to be involved in the

Figure 1.4 Chromosome 16 rearrangements associated with acute myeloid leukaemia

The normal chromosome 16 is shown and the breakpoint positions for the inv(16)(p13q22) are arrowed. The heterochromatin present in the long arm of the normal chromosome 16 (hatched area) assists in the identification of the long and short arms. The inv(16) chromosome associated with acute myeloid leukaemia, in particular the M4Eo variant can be difficult to identify in spreads of metaphase chromosomes derived from myeloid leukaemic cells. An experienced cytogenetic microscopist is required for confident diagnosis of this abnormality. The t(16;16)(p13;q22) events are also depicted in the figure demonstrating the short arm breakpoint on one chromosome 16 and the long arm breakpoint on its homologue. The del(16q22), associated with a morphologically identical leukaemia, may have prognostic significance for the patient with leukaemia containing this clonal karyotypic abnormality.

Chromosome 16

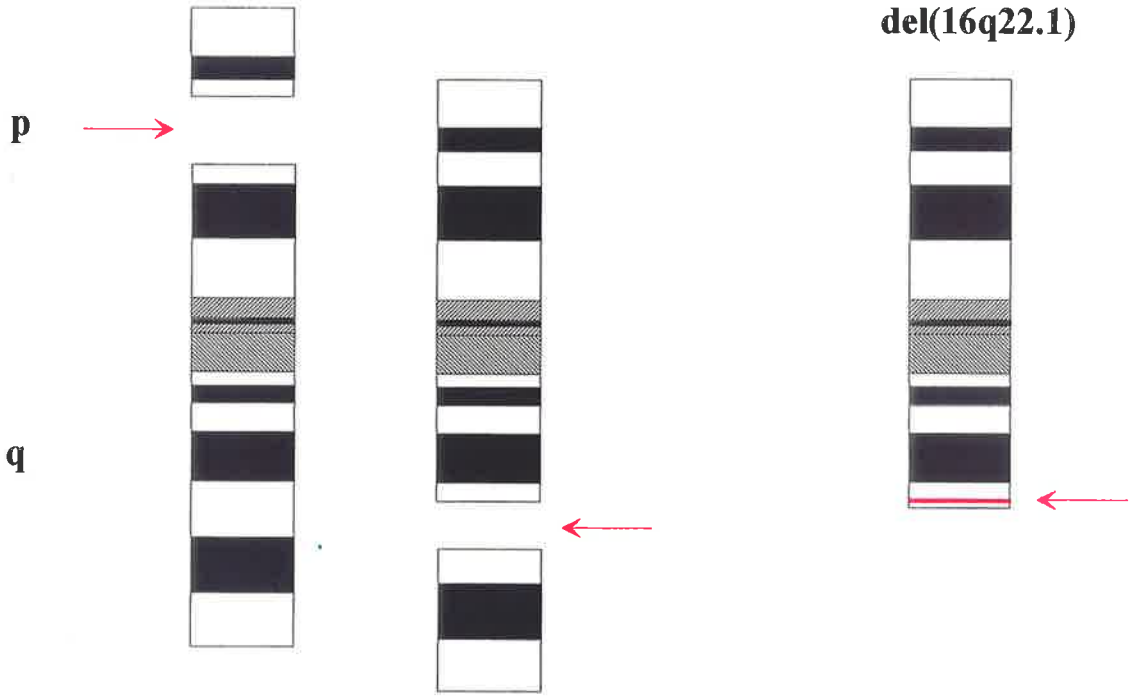
Inv(16)(p13.13;q22.1)



breakpoint positions arrowed

t(16;16)(p13.1;q22.1)

del(16q22.1)



Chromosome 16 homologues

pathogenesis of the M4 Eo variant by interfering with the utilisation of zinc and thereby granulocyte and monocyte differentiation. Subsequently, Sutherland (1990) demonstrated an intact gene complex in the standard inv(16) and remapped the gene complex to 16q13. The long arm breakpoint was remapped by Sutherland 1990 using fluorescent *in situ* hybridisation (FISH) techniques, to the proximal half of band 16q22.1 between the locus D16S4 and the HP gene. Dauwerse (1990) mapped the short arm breakpoint to band 16p13 by *in situ* hybridisation of anonymous DNA probes mapped to the region and sub-localised the breakpoint between two cosmids, cH16 and c36. Both of these cosmids were located distal to the rare folate-sensitive fragile site FRA16A and proximal to the PKD locus in the region 16p13.11-p13.3. This made it possible to more accurately, and with greater sensitivity compared with traditional banding techniques, detect the inv(16) abnormality associated with AML. As at this time, no candidate genes localised to these breakpoint regions (16p13.1 and 16q22.1), further mapping of anonymous cloned DNA would be required to narrow the regions of the long arm and short arm breakpoints. This was done using high resolution FISH and the chromosome 16 physical map established at this time (Callen 1989). Cloned DNA was available from a human chromosome 16 specific, cosmid library (Los Alamos National Laboratories).

1.4 Chromosome 16 physical map and hybrid panel

Chromosome 16 is selected for in tissue culture by use of the adenine phosphoribosyl transferase (APRT) gene at 16q24. Human cells, for example fibroblasts or bone marrow, can be fused with the mouse cell line A9, which contains a nonfunctional APRT gene, and grown in selective media. Hybrids can be identified which contain an intact APRT gene from the transfer of a human chromosome 16 or derivative chromosomes containing 16q24. This provides an

opportunity to isolate cancer breakpoints involving chromosome 16 in mouse/human somatic cell hybrids. For example, transfer of the inversion chromosome 16 to the A9 cell fulfils the criteria of an intact APRT gene and cytogenetic analysis can then be used to verify the absence of the normal human chromosome 16. Callen (1992), constructed an extensive mouse/human hybrid cell panel of chromosome 16 which contains more than 50 breakpoints and thus provides physical mapping at approximately 2Mb average resolution (Kozman 1993). Anonymous DNA probes and gene probes provide access to cloned DNA in each interval. This access can be further expanded since many of the mapped cosmid clones from Los Alamos National Laboratories are members of overlapping groups of clones known as cosmid contigs (Doggett 1995). Use of such hybrids in conjunction with detailed maps of chromosome 16 can provide a rapid mechanism for the positional cloning of breakpoints and subsequently the identification of the relevant genes. The band 16q22.1 is particularly rich in breakpoints, genes and cloned anonymous DNA segments. In 1992, it was estimated that the resolution of physical mapping in this region was in the order of 0.5 - 1.0 Mb. There were 17 probes within the region of interest, from which to identify flanking probes for use in the positional cloning of the long arm breakpoint of the inv(16). This formed the basis of the work described in chapter 3.

1.5 The use of positional cloning in the identification of leukaemia associated breakpoints

Chromosomal rearrangements, as found in acute leukaemias, provide an opportunity to examine disease related genes and improve understanding of the malignant processes involved in leukaemogenesis. The chromosome 8;21 translocation [t(8;21)(q22;q22)] was documented in 1973 and was the first translocation identified in AML (Rowley 1977). The

structural rearrangement involving chromosomes 15 and 17 [t(15;17)(q22:q12)], unique to acute promyelocytic leukaemia (APL), was identified in Rowley (1977). Both of these translocations have been analysed at the molecular level and provide examples of genetic abnormalities accompanying acute leukaemia.

APL is morphologically distinguishable from other subtypes of AML by the predominance of malignant promyelocytes in the bone marrow (myeloid differentiation being blocked at this stage) and by the presence of a severe haemorrhagic fibrinolytic state. In the majority of cases it is accompanied by the t(15;17) as detailed previously (table 1.1). The translocation breakpoints did not involve any of the candidate genes that had been localised to the breakpoint regions at this time and required a broader physical approach to the cloning of the breakpoint was required (Borrow 1990). The approach used will be discussed as a paradigm for approaches to positional cloning of oncogenes.

The approach used in this case involved pulsed field gel electrophoresis and the formation of an interspecies hybrid containing the region of chromosome 17 involved in the translocation (Borrow 1990, de Thé 1990). From this, a library of the human chromosome 17 region was constructed and flanking subclones identified. An HL60 (an acute promyelocytic cell line - Collins 1982) cDNA library was screened with unique fragments from these subclones and a unique transcript was isolated. This transcript (cDNA clone) identified a band shift, in DNA from acute promyelocytic leukaemic bone marrow cells digested with restriction endonucleases, and subsequent to this, the retinoic acid receptor was identified as the interrupted gene on chromosome 17. This finding was particularly interesting in light of the

clinical response of APL patients to high dose retinoic acid and the *in vitro* differentiation of APL promyelocytes seen on exposure to retinoic acid.

The gene disrupted on chromosome 15, subsequently labelled PML for promyelocytes (Kakizuka 1991), was found to belong to the zinc finger protein family, a novel class of transcription factors/DNA binding proteins. A fusion product PML-RAR was formed between the NH_2 terminus of the PML protein and the COOH terminus of the retinoic acid receptor. This product was found to be retinoic acid responsive and to display both promoter and cell specific differences from the wild type RARα. The PML-RAR fusion protein created a potentially bifunctional protein with multiple DNA-binding and transcriptional regulatory properties. It was superinducible by retinoic acid and its basal activity suppressed by the absence of retinoic acid (Kakizuka 1991). It was thought that the fusion protein may act in a dominant negative manner to block the actions of the wild-type PML. Addition of retinoic acid could reverse this inhibition by transforming the receptor to an activated state.

In the clinical setting, delivery of high dose all trans-retinoic acid results in the differentiation of the existing malignant promyelocytes but does not in itself cure the disease (Fenaux 1992). Chemotherapy is further required to abolish the malignant clone and improve overall survival (Wu 1993). The identification of the genes involved in this breakpoint thus provided insight into potential therapies at the patient level and further understanding into the pathways of myeloid differentiation .

A further example of the identification of genes involved in a translocation breakpoint is that of the t(8;21) of M2 acute myeloid leukaemias. The two genes involved are the *AML1* gene on chromosome 21 and *ETO* gene on chromosome 8 (Miyoshi 1991, Shimizu 1992). These form a chimeric gene on the derivative chromosome 8 and result in a chimeric transcript detectable by RT PCR (Nucifora 1993). The breakpoints were cloned through positional cloning using flanking anonymous DNA markers and pulsed field gel electrophoresis (PFGE). In this case, the two genes are again involved in transcription regulation, the AML1 gene being analogous to the PEPB2αB in the mouse (Bae 1993) and includes a domain with high homology to the runt gene which controls early developmental processes in *Drosophila melanogaster* (Erickson 1992). The cloning of this breakpoint has not led to any immediate changes in the therapeutic approach to this leukaemia however it provides information which may eventually result in the greater understanding of the disease biology.

1.6 Multidrug resistance and P-glycoprotein

The phenomenon of multidrug resistance (MDR) was first described by Kessel (1970) who noted that cultured P388 murine leukaemia cells selected for resistance to vinblastine, were also cross resistant to actinomycin D, daunorubicin, and the vinca alkaloids. This experimental phenotype represented a cross resistance to a number of hydrophobic compounds that were otherwise structurally unrelated. Other investigators found similar cross resistance developing in cultured Chinese hamster lung and ovary cells (CHO) and also noted the overexpression of an integral membrane glycoprotein of approximately 170kDa, which was absent in the drug sensitive parental CHO cells. The term multidrug resistance (MDR) phenotype was coined implying the development of cross resistance to multiple natural product antineoplastic agents

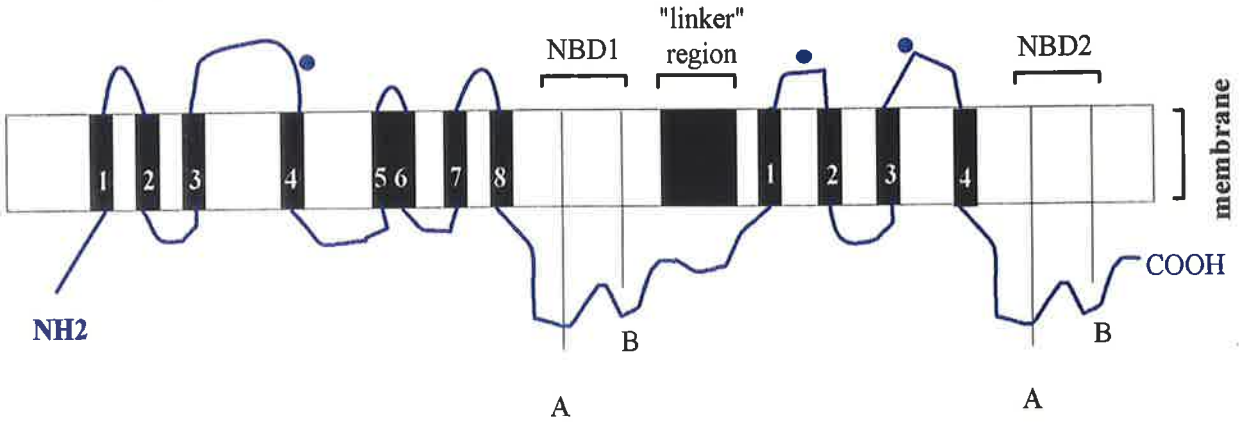
after exposure to only one of these drugs (Ling 1982). The protein found to be associated with the development of the MDR phenotype was termed P-glycoprotein by Ling (1982, 1983) and represents the protein product of the *MDR1* gene, found in humans and rodents. This gene is part of the ATP binding cassette (ABC) superfamily of transport proteins and is located on the long arm of chromosome 7 (7q21.1). The ABC superfamily can be divided into two groups, based on amino acid sequence and structure. One group contains P-glycoproteins, the major histocompatibility complex (MHC) class II-linked peptide transporters, the bacterial exporters (Eco/HlyB and Pas/LktB), the heterocyst differentiation protein (Ana/HetA), the malarial parasite transporter (Pfa/Mdr1), and the yeast mating factor exporter (Ysc/Ste6). The other group contains the multidrug resistance protein (MRP), anthracycline resistance associated protein (ARA), the leishmania P-glycoprotein related molecule, and the Cystic Fibrosis Transmembrane conductance Regulator proteins (CFTR) (Cole 1992).

Common to all of this ABC family of transporters is transmembrane region which spans the membrane multiple times in succession and a cytoplasmic nucleotide (ATP) - binding domain, but they otherwise demonstrate considerable structural diversity. P-glycoprotein consists of 1280 amino acids organised in two tandem repeats each consisting of 610 amino acids, joined by a linker region of 60 amino acids. Each repeat consists of an NH_2-terminal hydrophobic domain containing six potential transmembrane helices, followed by a hydrophilic domain containing a nucleotide binding site. The most accepted topological arrangement for this protein, as predicted by computer assisted hydropathy and consensus motif analysis of the nucleotides and deduced amino acid sequence analysis, is depicted in figure 1.5 (Chen 1986).

Figure 1.5 Predicted topology for P-glycoprotein and MRP

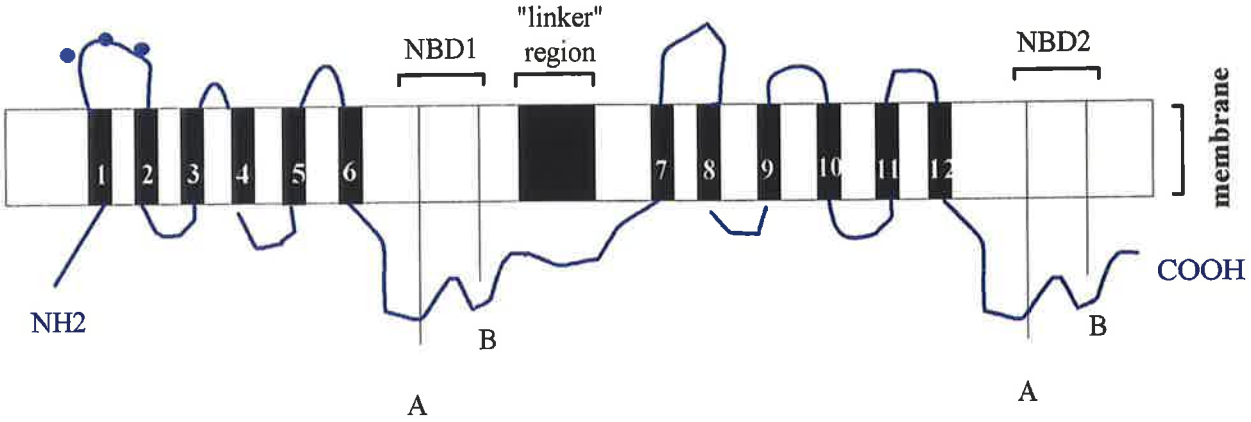
Computer-assisted hydropathy and consensus motif analyses of the nucleotide and deduced amino acid (aa) sequence of P-glycoprotein and MRP suggests that they are most likely comprised of two highly similar halves, each containing a nucleotide binding domain (NBD) connected by a linker sequence. They contain 12 transmembrane domains in a six plus six configuration for P-glycoprotein (#1-#12) and probably an eight plus four configuration for MRP. The most highly conserved regions between the two molecules lie within the NBD regions surrounding the Walker A (A) and Walker B (B) motifs. The symbol (•) represents glycosylation sites for the two proteins. (After *Cole, SPC.* **Lung Cancer: Principles and Practice** Lippincott, Philadelphia 1995).

Multidrug Resistance Protein



1531 aa

P-glycoprotein



1280 aa

The substrates for the ABC proteins are extremely varied, ranging from chloride ions (as in the case of CFTR's), to the complex peptides involved in the MHC class II transporters. The list of substrates for P-glycoprotein, table 1.2, include colchicine, actinomycin-D, daunorubicin (anthracyclines), epipodophyllotoxins, taxol and the vinca alkaloids (Bellamy 1990, Campos 1992). Binding of these drugs to P-glycoprotein has been demonstrated through photoaffinity studies and mutational analysis (Cornwell 1986, Currier 1989, Loo 1994). However, the mechanism(s) by which the physical transport of these compounds through the membrane occurs has not been delineated and there are many questions still unanswered concerning the apparently small changes in cytosolic drug accumulation and therefore transport of the compound through the plasma membrane and the apparent significant increase in drug resistance demonstrated by the cell. Several models have been proposed to account for multidrug resistance. Firstly, a change in drug accumulation in the drug resistant cell due to reduced drug influx as well as increased drug efflux. An ATP-driven pump such as P-glycoprotein is proposed to act as a flippase and expel the drug from the lipid bilayer (Higgins 1992b). The problems encountered with this model concern the inconsistencies in the efficiency of the pump verses the degree of drug resistance evident, as already stated. One proposal is that drugs are compartmentalised away from the nucleus remaining within the cytosolic compartment suggesting that the nuclear concentration may be a more important measurement of drug exposure than the cytosolic drug concentration (Simon 1994b).

Other theories for drug resistance propose that physical changes within the resistant cell are important in the development of drug resistance, such as intracellular pH (Simon 1994a). The typical drugs involved in the *in vitro* phenotype of drug resistance are weak bases, and in their

Table 1.2 Multidrug resistance & P-glycoprotein: antineoplastic and resistance reversing agents

Antineoplastic agents to which multidrug resistant (MDR) cells are resistant are listed in the top table. Low levels of cross resistance to some agents is noted against topotecan, mithramycin and mitomycin C. Idarubicin is now known to be included in the cross resistance seen in leukaemic cells with the classic drug accumulation defect. The second table is a list of many of the agents noted to reverse P-glycoprotein associated multidrug resistance. The method of action of these drugs is unlikely to be the same for each class of drugs. The data is predominantly derived from *in vitro* research.

Antineoplastic agents to which MDR cells are resistant

Vinca Alkaloids: vinblastine
vincristine
navelbine

Anthracyclines: doxorubicin
daunorubicin
epirubicin
idarubicin*

Taxanes: paclitaxel
taxotere

Epipodophyllotoxins: etoposide
teniposide

Other: mitoxantrone
dactinomycin
amsacrine
trimetrexate
topotecan†
mithramycin†
mitomycin C†

* previously reported as being effective against MDR lines (Petrini1993), evidence now suggests that MDR lines are cross resistant to idarubicin with the classic accumulation defect of P-glycoprotein.

†low levels of cross resistance to these agents

Selected pharmacologic agents with ability to reverse multidrug resistance#

Calcium Channel Blockers: verapamil
nifedipine
diltiazem
PAK-200
Ro11-2933
niguldipine

Calmodulin Antagonists: trifluperazine
prochlorperazine
fluphenazine
trans-flupenthixol

Vinca Alkaloid Analogues: vindoline
thaliblastine

Steroidal Agents: progesterone
tamoxifen
toremifene
megestrol acetate
17β-estradiol glucuronide

Immunosuppressive Drugs: cyclosporin A
SDZ PSC 833
SDZ 280-446
FK506
rapamycin

Antibiotics: cephoperazone
ceftriaxone
erythromycin

Miscellaneous compounds: dipyridamole
quinidine
chloroquine
terfenadine
reserpine
amiodarone
methadone
tolyphorphin

predominantly *in vitro* data

neutral form are hydrophobic and diffuse across the plasma membrane. Drug resistant cells tend to be more alkaline than drug sensitive cells. This prevents protonation of the drug in the drug resistant cell, reduces the affinity for the DNA/RNA and tubulin targets and would reduce drug accumulation relative to their drug sensitive counterparts. Transport into endoplasmic vesicles may also be affected both by pH and active transport molecules (Simon 1994a). These processes can influence the concentration of intracellular active drug which in turn can influence rates of drug efflux. Other mechanisms of drug resistance include non P-glycoprotein factors such as glutathione-S, topoisomerase II inhibition, an unidentified 110kDa protein associated with reduced drug accumulation and MRP (Simon 1994b, Scheper 1993). These may be equally important in the overall picture of drug resistance, each one assuming greater or lesser importance in any one cancer cell type.

1.7 MRP - Structure and function

The mRNA for a new multidrug resistance protein, MRP, was cloned from the human small cell lung cancer cell line, H69AR (Cole 1992). MRP and P-glycoprotein are members of the ATP-binding cassette superfamily of transmembrane transporters (figure 1.5). However these two proteins have only 15% amino acid structural homology (Cole 1993). Like P-glycoprotein, in *in vitro* experiments MRP has been shown to confer resistance to a spectrum of structurally diverse, natural and synthetic products (Cole 1994), including daunorubicin, a major component of the chemotherapeutic regimen which has been most widely used in the treatment of AML. MRP is encoded by a 6.5kb mRNA and expression is detectable by Northern analysis of RNA in many normal cell types including the gastro-intestinal tract, kidney, hepatocytes, lymphocytes and normoblasts as well as other early haematopoietic

precursors ($CD34^+$) and muscle (Krugh 1995). In comparison to these cell types, there is relatively high level of expression in lung, thyroid, testis, bladder and adrenals (Zaman 1993). Overexpression of MRP mRNA and protein has been found in a number of multidrug resistant cell lines, including the human leukaemia cell line, HL60/ADR (Marquardt 1992, Krishnamachary 1994) and the murine erythroleukaemia cell line, PC-V160 (Slapak 1994). The MRP gene, which has been shown to be amplified in some of the cell lines where it is overexpressed, has been mapped to chromosome 16 at band p13.1, close to the short arm breakpoint of inv(16) (Cole 1992, Slovak 1993).

In situ hybridisation studies and examination of membrane subfractions reveals that MRP is a 1531 amino acid, ATP binding, integral membrane glycophosphoprotein with an apparent molecular weight of 190kDa. Analysis of the subcellular distribution of MRP confirms that it may be plasma membrane bound or integral to subcellular membranes and that there are different intracellular distributions of the protein in different cell types (Almquist 1995, Barrand 1993). Recent data suggests that its biological function may be that of an ATP-dependent pump for leukotriene C_4 (LTC4) and structurally related amphiphilic compounds, including glutathione-S-conjugates of xenobiotics, as seen in the canalicular membrane of the liver (Leier 1994). It has also been noted that the leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance, providing further evidence of a role for MRP in the transport of leukotrienes (Gekeler 1995b). Another study has shown expression of the *MRP* gene in human liver canaliculi and hepatocytes with evidence of a glycosylated 190kDa protein reactive with MRP polyclonal antibodies (Mayer

1995) This study also demonstrated high affinity transport by MRP of LTC4 as a glutathione S-conjugate.

The evidence that MRP acts as an ATP-dependent plasma membrane pump is increasing. However, both decreases in drug accumulation and unaltered accumulation have been reported in various cell types where there is overexpression of MRP (Cole 1994, Zaman 1994, Eijdems 1995, Breuninger 1995). This apparent inconsistency may be explained by differences in intracellular localisation of the protein, with plasma membrane bound MRP decreasing drug accumulation while that fraction on internal membranes promoting sequestration, thus preventing the drug from reaching its nuclear target. For example, in the H69AR cell line(small cell lung cancer, drug resistant cell line), marked alterations in intracellular drug distribution with little net change in intracellular drug concentration have been associated with increased MRP expression (Cole 1991). While in HL60/ADR cells (acute promyelocytic leukaemic cell line) rapid removal of drug from the nucleus to the extracellular space accounts for drug resistance (Marquardt 1992). Analysis of drug kinetics using radiolabelled daunorubicin, revealed decreased accumulation and increased efflux of the daunorubicin while confocal microscope analysis of the cells revealed an altered pattern of intracellular drug distribution. This was characterized by the initial accumulation of drug in a perinuclear location, followed by the development of a punctate pattern suggestive of a drug sequestration process (Breuninger 1995).

It is presently unknown whether MRP pumps xenobiotics in a manner analogous to that proposed for P-glycoprotein, or whether it influences drug accumulation and/or intracellular

distribution using intermediary vehicles. The mechanism is likely to differ, as MRP associated MDR is not significantly reversed by agents which reverse P-glycoprotein related MDR, such as verapamil, but is reversed by other compounds not known to reverse P-glycoprotein related MDR (Gekeler 1995a, Versantvoort 1994). This is despite the similar drug resistance profiles for MRP and P-glycoprotein expression. It may be that MRP transports GSH S-conjugates and is closely related to the GS-X pump (Müller 1994). This model helps to explain the effects of buthionine sulphoximine-mediated sensitivity in MRP overexpressing cell lines (Schneider 1994). Buthionine sulphoximine causes depletion of intracellular glutathione and results in complete reversal of resistance to doxorubicin, daunorubicin, vincristine and VP16 in lung cancer cells transfected with a MRP cDNA expression vector (Zaman 1995). However drugs such as doxorubicin and vincristine were not previously known to undergo any modification to make them suitable substrates for the GS-X pump. It may be that MRP may be more versatile in its mode of action than P-glycoprotein.

The exact phenotype for MRP related MDR is uncertain due to the coexpression of multiple resistance mechanisms such as topoisomerase II and the recently cloned Anthracycline Resistance Associated protein, ARA (Cole 1994, Longhurst 1996). However, attempts to distinguish the cellular effects of MRP vs P-glycoprotein have been made by a number of investigators. Our current understanding of MRP function has been determined by the use of cells transfected with *MRP* expression vectors and by the investigation of drug selected cell lines overexpressing the MRP gene and protein. HeLa cells transfected with *MRP* cDNA and expressing vector encoded *MRP* mRNA and 190kDA encoded protein show a 5-15 fold

increase in doxorubicin and daunorubicin resistance as well as a similar level of cross resistance with vincristine and VP16 (Grant 1994, Cole 1994). This demonstrated a functional correlation of the expression of MRP and the resultant level of drug resistance. A second study utilising transfected non-small cell lung cancer, SW-1573/S1 cells, showed a similar phenotype of drug resistance and a similar decreased steady state accumulation of drug due to an active transportation of the drugs across the plasma membrane (Zaman 1994). Breuninger (1995) demonstrated increased resistance to doxorubicin, daunorubicin, etoposide, actinomycin D, vincristine and vinblastine in *in vitro* experiments using NIH/3T3 transfected with cognate MRP cDNA. No increased resistance to taxol was seen, distinguishing the resistance phenotype of the cells from a P-glycoprotein related phenotype. A study by Feller (1995), investigated the functional effects of P-glycoprotein and MRP using Rhodamine 123 and daunorubicin combined with specific modulators of the two proteins. This was analysed by flow cytometry and demonstrated the relative contributions of MRP and P-glycoprotein to the MDR phenotype in a number of drug resistant cell lines. Therefore, although there is a degree of crossover between the MDR phenotypes arising from P-glycoprotein and MRP expression, it may be possible to determine the contributions of each protein to the expression of multidrug resistance in any one cell.

It is likely that within one cell, more than one multidrug resistance protein is functioning. Co-expression of multidrug resistance proteins has been demonstrated in one study by Brock (1995), where a human small cell lung cancer cell line H69/VP developed by continuous exposure to increasing doses of VP16 demonstrated sequential co-expression of *MDR1* and *MRP*. Double immunocytochemical staining of single cells demonstrated co-expression of

the two proteins and RT-PCR analysis of cellular RNA demonstrated that overexpression of *MRP* occurred prior to *MDR1*. Sequential over-expression was also demonstrated in a study by Zhou (1996), K562 cells were exposed to increasing levels of homoharingtonine (a plant alkaloid derived from the bark of the Chinese evergreen tree) resulting in a series of drug resistant cell lines. It was shown that *MRP* expression occurred first, followed by a period of dual expression. However, at the highest concentration of drug exposure, a 20 fold increase in *MDR1* was demonstrated, at which time gene amplification of *MDR1* was noted and at this time, *MDR1* expression predominated. Eijdems (1995) noted that high levels of drug resistance were associated with MRP gene amplification whereas low level drug resistance appeared to be due to transcriptional activation. In the clinical setting, the exposure of leukaemic cells to cytotoxic agents is not of the same order of magnitude as the *in vitro* exposure of leukaemic cell lines. Consequently, gene amplification has not been associated with over-expression of the *MRP* gene, however transcriptional activation may be an underlying mechanism for the increased expression of MRP seen in relapsed AML patients (Schneider 1995) and was postulated in a study by Burger (1994b). Clinical studies are required to relate the *in vitro* findings to *in vivo* cellular functions and to establish if MRP plays an important role in the clinical as well as experimental multidrug resistance phenotype. The use of monoclonal antibodies to unique epitopes of MRP should assist the functional analysis of this protein (Flens 1994, Hipfner 1994).

1.8 Biological significance of multidrug resistance in leukaemia

1.8.1 Drug Resistance Proteins and Resistance Reversing Agents

Drug resistance presents a significant clinical problem in the treatment of acute leukaemias, however while some studies show increasing levels of P-glycoprotein or its cognate mRNA, correlate with increasing drug resistance seen in these diseases, the evidence is by no means uniform. It is possible that P-glycoprotein expression is simply one marker of poor prognosis seen with CD34+ leukaemias (Abbaszadegan 1994, te Boekhorst 1995) or that other drug resistance factors are acting within the primitive leukaemic cell types. For example, it has been demonstrated *in vitro* that anthracycline selected multidrug resistance derivatives of leukaemic cell lines can overexpress MRP (Krishnamachary 1994) and increased MRP expression has been documented in a number of previously untreated leukaemias as well as relapsed leukaemias (Zhou 1995). The reduced expression of topoisomerases seen in drug resistant leukaemias has also been investigated and found to be commensurate with an increase in MRP and P-glycoprotein expression in the course of relapsed ALL (Beck 1995). There are other recently described proteins such as LRP: Lung-Resistant Protein (Izquerido 1996), ARA(Longhurst 1996), 110kDa protein (Scheper 1993) all of which appear to have multidrug resistance properties. The expression and function of each of these proteins needs to be investigated simultaneously in examples of clinical drug resistance, to determine their relative contributions to the drug resistance seen following exposure to the cytotoxics used in the treatment of acute leukaemia.

Although *MRP* is known to be expressed in a number of terminally differentiated haematopoietic cell types, two studies examining drug resistant acute myeloid leukaemias

found that expressed *MRP* mRNA levels did not differ significantly from that of normal haematopoietic cells (Hart 1993, Abbaszadegan 1994). Ross (1996) examining leukaemic blasts, used a quantitative RT-PCR assay to demonstrate a 1.7 fold increase in *MRP* expression above a drug sensitive cell line. This increase in *MRP* gene expression correlated with a lower mean accumulation of daunorubicin within these cells compared with those cells expressing relatively less *MRP*. Therefore correlating the expression of *MRP* with a functional change in the level of drug accumulation. Other studies have looked at a variety of leukaemias for the expression of MRP and found increased expression in some portion of the cohort studied. For example, Burger (1994a) looked at acute and chronic leukaemias and found elevated levels of *MRP* mRNA in one of nine untreated patients and two of seven patients who had previously received chemotherapy for AML. In this study, chronic lymphocytic leukaemia patients including both naive and previously treated cases, 71% (15 out of 21 patients) had relatively high levels of *MRP* mRNA detectable. Hart (1994) found a significant increase in expression of *MRP* in ALL cells compared with both normal peripheral blood mononuclear cells and with *de novo* AML cells. This study also found the expression of *MRP* was higher in the relapsed AML patients studied than in the *de novo* AML patients. This finding was repeated in the study by Zhou (1995).

The conclusions to be drawn from these studies is that a low level of expression of MRP occurs in a wide range of cell types and it would appear that relatively small increases in expression result in differences in the drug accumulation within the cell. This must now be correlated with the functional drug resistance resulting from the changes in intracellular drug accumulation or drug redistribution. It is probable that increased drug resistance will be seen

in relapsed AML cells over *de novo* AML cells. However, the mechanism by which this occurs is not fully determined and a number of mechanisms may be acting simultaneously.

Although the spectrum of drugs to which MRP and P-glycoprotein confer resistance is similar, the phenotypes of the cells overexpressing each of the proteins may differ in some important respects. The overexpression of P-glycoprotein results in reduced net drug accumulation and this is reversed by drugs such as cyclosporin A and verapamil as demonstrated by *in vitro* studies (Tsuro 1989, Chambers 1989). The ability of these drugs to reverse MRP mediated resistance appears variable (Cole 1994, Zaman 1994). Other studies have found almost complete reversal of drug resistance to vincristine, etoposide and doxorubicin in T98G cells which overexpress MRP using either verapamil or NIK250 (a dihydropyridine analogue) (Abe 1995). Clinical trials are currently in progress to investigate the efficacy of drugs such as cyclosporin A and verapamil as a means of reversing the drug resistance seen with the expression of P-glycoprotein in leukaemic patients (Fisher 1995). Those phase I & II studies already completed show mixed results and clearly demonstrate that there is more than one type of drug resistance mechanism occurring in the cancer cells of different leukaemias at any one time (table 1.3). A tyrosine kinase inhibitor, genistein, has been shown to specifically inhibit MRP mediated drug resistance and appears to competitively inhibit the transport of daunorubicin by MRP and to reverse the effects of MRP mediated resistance for other drugs (Versantvoort 1994, Nagasawa 1996, Takeda 1994). A PKC (protein kinase C) inhibitor has also been shown to modulate drug resistance due to MRP without effecting gene expression (Gekeler 1995a), presumably acting on the phosphorylation status of the protein, shown to be of importance to drug transport by Ma

Table 1.3 Clinical trials utilising resistance reversing agents in AML

Modulator (Serum Concentration)	**Cytotoxin(s)**	**Tumour Type** (No. Patients)	**Comments**	**Author**
Verapamil (iv) (≈1 μM)	Vinblastine and VP-16	Paediatric (7) (5 leukaemic)	Six with transient minor responses; mean course per patient: 1.6.	Cairo 1989
Dexniguldipine (po) (≈ 0.3 μM)	Daunorubicin and cytarabine	AML (16)	All dauno/cytarabine refractory patients; 3CRs; mild toxicity	Scheulen 1994
Dexniguldipine (iv) (≈ 0.5 μM)	Vinblastine	AML (26)	Dysrhythmias were dose limiting; no $PK_{vinblastine}$ reported	Scheulen 1994b
CsA (iv) (1.2 μM)	Daunorubicin and cytarabine	AML (42)	26 CRs and 3 PRs; increased dauno-rubicin levels and haematologic toxicity	List 1993

Information on studies involving resistance reversing agents incorporated into the treatment regimen of AML patients is shown. The study by Cairo(1989) also included two paediatric patients with solid tumour malignancies. Intravenous (iv) and oral (po) routes of administration were used. Complete responders (CRs) and partial responders (PRs) are listed. CsA = cyclosporin A. VP-16 = etoposide. These studies represent phase I and II clinical trials of resistance modifying agents for the purpose of reversing P-glycoprotein related MDR.

(1995). The exact mechanism of action of these reversing agents is not known, neither is the mechanism by which the drugs are transported through the membrane. In fact, it is evident that different anthracycline derivatives appear to be handled differently by the multidrug resistance proteins despite their obvious structural similarities (Ross 1995, Nagasawa 1996). However, it is likely that identification of the proteins responsible for conferring drug resistance and the mechanism of transport of drug from one compartment to another will provide important information for the optimal design of effective treatment protocols that combine resistance reversing agents and chemotherapeutic drugs.

1.8.2 Prognostic Implications

Patient survival data correlated to specific expression of the MDR factors is largely inconclusive and incomplete however drug resistant phenotypes appear to be the major cause of recalcitrant disease. A recent study of acute leukaemia (Schuurhuis 1995), suggests that the combined overexpression of P-glycoprotein/*MDR1* and *MRP*, together with Arabinofuranosyl-cytosine sensitivity may best predict clinical response to chemotherapy. While another study suggests that in relapsed acute lymphoblastic leukaemia, topoisomerase II levels as well as MRP/P-glycoprotein expression vary significantly (Beck 1995). te Boekhurst (1995) correlated failure to achieve complete remission from acute myeloid leukaemia, with autonomous growth of the leukaemic cells, CD34 and P-glycoprotein expression, both alone and simultaneously, but did not find a correlation with expression of a p110 protein associated with drug resistance, MRP expression, or incorporation of Ara-C in the cell. More recent work with a new protein (LRP) known to be a membrane component of the newly identified subcellular structure referred to as "vaults", currently has a predictive

inference on the outcome of certain solid tumour cancers and possibly leukaemias (Izquerido 1996). It is likely that clinical multidrug resistance phenotypes represent a combined picture of the various mechanisms of drug resistance operating in consort and that in haematological malignancies as well as solid tumours, each of these mechanisms will need to be evaluated to fully understand the biology of the disease.

The preliminary reports referred to above must be extended by prospective studies in which the levels of MRP mRNA and, most importantly, protein are correlated with functional studies of drug resistance. Determination of the levels of MRP as well as its cognate mRNA are essential since both may not change concordantly. Until recently, determination of protein levels has not been possible. Fortunately, monoclonal antibodies to MRP have now been described which appear suitable for clinical studies (Almquist 1995, Hipfner 1994). Such prospective studies are essential before any definitive statements concerning the role of MRP in leukaemic cell drug resistance can be made.

MATERIALS AND METHODS

Chapter 2

2.1 Introduction

Most of the methods described in this Chapter were well established and used routinely in the Department of Cytogenetics and Molecular Genetics at the Women's and Children's Hospital (Adelaide, Australia). Only materials and methods used in more than one chapter will be presented here, otherwise, they will be described in their corresponding chapters. All enzymes were obtained from commercial sources and were used in accordance with the manufacturer's specifications. All chemicals and solvents were of analytical grade.

2.2 Materials

2.2.1 Enzymes

The following enzymes were obtained from:

Calf Intestinal Alkaline Phosphatase	Boehringer Mannheim, Germany
Deoxyribonuclease I	Sigma Chemical Co., St. Louis, M, USA
E.Coli DNA Polymerase I (Klenow Fragment)	Amersham, Australia Pty Ltd
Lysozyme	Boehringer Mannheim
Not I Methylase	New England Biolabs Inc., MA, USA
Proteinase K	Sigma Chemical Co.
RNase A	Boehringer Mannheim
Sequenase	USB (United States Biochemical Corp.)
Superscript(RNase H^- reverse transcriptase)	Gibco BRL, Life Technologies
T4 DNA Ligase	Boehringer Mannheim

T4 Polynucleotide Kinase	Pharmacia, Uppsala, Sweden
Taq Polymerase	Boehringer Mannheim

All restriction endonucleases were from New England Biolabs (Beverly, M., USA)

2.2.2 Electrophoresis

The reagents were obtained from the following companies:

Acrylamide	Ajax, Auburn NSW, Australia
Agarose - Type N.A	Pharmacia
Low Gelling Temperature Seaplaque	(FMC)
Pulsed Field Grade	Biorad
Ammonium Persulphate	Ajax
Bromophenol Blue	B.D.H. Chemicals LTD, Pool,England
Ethidium Bromide	Boehringer Mannheim
N,N,Nl,Nl-tetramethylethylene diamine (TEMED)	Biorad, Richmond California USA
Urea	Ajax
Xylene Cyanol	Tokyo Kasei, Tokyo, Japan

<u>Molecular weight markers</u>

SppI	Bresatec
DRIgest TM III	Pharmacia
Lambda-PFGE	Pharmacia
Yeast-PFGE	Pharmacia

2.2.3 Radiochemicals

alpha -^{32}P-dCTP, 3000 Ci/m.mole	Radiochemical Centre, Amersham
gamma-^{32}P-ATP, 5000 Ci/m.mole	Radiochemical Centre, Amersham

2.2.4 Buffers and solutions

Buffers and solutions routinely used in this study were as follows:

Formamide Loading Buffer
92.5 % (v/v) formamide
20mM EDTA
0.1% (w/v) xylene cyanol
0.1% (w/v) bromophenol blue

lOx Loading Buffer
50% (v/v) glycerol
1% (w/v)SDS
l00mM EDTA
0.1% xylene cyanol
0.1% (w/v) bromophenol blue

lOx Ligation Buffer
0.5M Tris-HCl(pH7.4)
0.lM $MgCl_2$
0.lM dithiothreitol
l0mM spermidine
l0mM ATP
lmg/mL bovine serum albumin

M9 salts	1.05 K_2HPO_4
	0.45% (w/v) KH_2PO_4
	0.1% (w/v) $(NH_4)_2S0_4$
	0.05% (w/v) sodium citrate
Phosphate Buffered Saline (PBS)	130mM NaCl
	l0mM $NaHPO_4$
	l0mM NaH_2PO_4, pH7.2
2xPCR mix	33mM $(NH_4)_2S0_4$
	133mM Tris-HCl
	2% (v/v)JB-mercaptoethanol
	13mM EDTA
	0.34mg/ml BSA
	20% (v/v) DMSO
	3mM dATP, dGTP, dTTP, dCTP.
SE	75mM NaCl
	25mM EDTA pH 7.5
20xSSC	3M NaCl
	0.3M tri-sodium citrate
	adjust with NaOH to pH7.0

TAE	40mM Tris-acetate
	2mM EDTA, pH8.5
TBE	89mM Tris-base
	89mM boric acid
	2.5mM EDTA, pH 8.3
TE	l0mM Tris-HCl (pH 7.5)
	0. lmM EDTA
TES	25mM Tris-HCl (pH 8.0)
	l0mM EDTA
	15% (w/v) sucrose
TSB	10% (w/v) polyethelyene glycol
	(PEG) mw 3600
	5% (v/v) DMSO
	100mM $MgCl_2$
	l00mM $MgS0_4$
	L-Broth to appropriate volume

2.2.5 Bacterial media

2.2.5.1 Liquid media

All liquid media were prepared using millipore water and were sterilised by autoclaving. The compositions of the various media were as follows:

AHC medium	0.67% (w/v) yeast nitrogen base
	w/o amino acids
	1% (w/v) casein hydrolysate-acid
	0.006% adenine
	2% glucose
L-Broth	1% (w/v) Bacto-tryptone
	0.5% (w/v) Bacto yeast extract
	1% (w/v) NaCl, pH to 7.5 with NaOH.
Minimal Medium	lmM $MgS0_4$
	0.1mM $CaCl_2$
	lmM thiamine-HCl
	0.2% (w/v) glucose
	1 x M9 salts
2 x YT	1.6% (w/v) Bactotryptone
	1% (w/v) Bacto yeast extract
	0.5% (w/v) NaCl

2.2.5.2 Solid media

L-Agar	L-broth 1% (w/v) Bacto agar
L-Amp	L-broth 1% (w/v) Bacto agar ampicillin (100μg/ml)
L-Kanamycin	L-broth 1% (w/v) Bacto agar kanamycin (50μg/ml)

2.2.6 Antibiotics

Ampicillin	Sigma
Kanamycin	Boehringer Mannheim
Tetracycline	Sigma

2.2.7 Bacterial strains

The E. coli strains used in this project are listed below:

XLI-Blue genotype: rec Al, end Al, gyr A96, thi-l, hsd R17, sup E 44, rel Al, lac[FIproAB, lac lqZAMI5, tn 10 (tetr)].

LE392 genotype: hsdR514, supE44, supF58, lac Y1, galK2, galT22, metB1, trpR55, mcrA.

2.2.8 Vectors

The vectors used in this study are the following:

Filamentous Phage:	
M13mpl8	Boehringer Mannheim
M13mpl9	Yanish-Perron *et al* (1985)
Phagemid Vectors:	
pBluescript SK II	Stratagene, La Jolla, California USA
Plasmid Vectors:	
pUC19	Bresatec, Yanish-Perron *et al* (1985)
pGEM11Zf(-)	Promega
Cosmid Vectors:	
Scos1	Stratagene, La Jolla, California USA

2.2.9 Miscellaneous materials

Dialysis tubing	Promega
Hybond N+™ Nylon Membrane	Amersham
NAP™-10 Column	Pharmacia Biotech
Sephadex G-50	Pharmacia P-LBiochemicals

T.A. Cloning Kit	Invitrogen
X-ray film	Kodak
TRIzol Reagent	Gibco BRL Life Technologies

2.2.10 Miscellaneous fine chemicals

5-bromo-4-chloro-3-indolyl-B-D galactoside	Boehringer Mannheim
Chemicals for Oligonucleotide Synthesis	Applied Biosystems
Cesium chloride	Boehringer Mannheim
Deoxynucleotides	Boehringer Mannheim
Dideoxynucleotides	Boehringer Mannheim
Dideoxysequencing Kits	USB
Dimethylsulphoxide (DMSO)	Sigma Chemical Co.
Isopropyl thio-B-D-galactoside (IPTG)	Boehringer Mannheim
Random Priming Oligolabelling Kits	Amersham
Phenol	Wako
Salmon-sperm DNA	Calbiochem
Sarkosyl	Ciba Geigy, Basle, Switzerland
SDS	Sigma Chemical Co
Spermidine	Sigma Chemical Co
Taq Dye DeoxyTM Terminator Cycle Sequencing Kit	Applied Biosystems

2.2.11 Collection of inv(16) leukaemic samples

Diagnostic inv(16) leukaemic samples and some remission samples from the same patients were obtained from Dr Cheryl Willman (Albuquerque, New Mexico). Other samples were sought from local sources including South Australian haematology laboratories and Australian cytogenetic laboratories. The diagnoses of the New Mexico samples were reviewed by a designated haematologist and cytogeneticist according to the South Western Oncology Group (SWOG) protocols. Treatment and survival data was also available through SWOG on each patient. Diagnostic bone marrow aspirations from local sources were reviewed by the student for confirmation of FAB subtype classification. The cytogenetic profiles were mostly performed by this laboratory and were reviewed with regard to the presence of an inverted chromosome 16, at the time of FISH analysis.

2.3 Methods

2.3.1 DNA isolation

2.3.1.1 Large scale isolation of plasmid DNA and cosmid DNA

(modification of Sambrook 1989)

10ml Luria Bertoni (LB) medium containing ampicillin (50μg/ml) was inoculated with a single fresh bacterial colony. The culture was incubated at 37°C for 5-7 hours with vigorous shaking, and then transferred to 100ml LB containing ampicillin (50μg/ml). After overnight incubation at 37°C with vigorous shaking, the culture was transferred to two 50ml centrifuge tubes. The tubes were left on ice for 15 minutes and then spun at 3000 rpm for 15 minutes in a Jouan CR3000 centrifuge at 4°C. The supernatant was discarded and the cell pellet was gently resuspended in 300μl TE and glucose containing 60μl of 80mg/ml lysozyme. The

cell suspension was left at room temperature for four minutes and on ice for one minute. 1.2mls of 0.2M NaOH/1% SDS was added to the cell suspension, gently mixed and incubated on ice for a further five minutes. 900µl of ice cold 3M potassium acetate (pH4.3) was then added, and mixed by inversion. After resting on ice for 10 minutes, the lysed cell debris was cleared by centrifugation in a Beckman J2-21M/E centrifuge with a JA20 rotor at 15,000rpm for 15 minutes. The supernatant was mixed with 5.5 mls of ethanol and after five minutes at room temperature, precipitated nucleic acids were pelleted by centrifugation at 15,000rpm for 15 minutes. The DNA pellet was washed twice in 2ml of 70% ethanol, air-dried and resuspended in 200µl TE. To eliminate RNA in the DNA preparation, 10µl of 1mg/ml RNase was added to the DNA solution and incubated at 37°C for one hour. To eliminate proteins in the DNA preparation, 100µl of 3x proteinase K buffer, 10µl of 10% SDS and 2µl of 10mg/ml proteinase K were added to the DNA solution and incubated for one hour at 37°C. Following incubation, the DNA was phenol extracted, ethanol precipitated and dissolved in 200µl of TE. If required the DNA was purified by banding on a CsCl gradient.

2.3.1.2 Small scale isolation of plasmid DNA

(modification of Birnboim 1979)

A single bacterial colony was inoculated to 1.5ml of LB medium containing ampicillin (50µg/ml) in a 10ml tube. The culture was incubated at 37°C overnight with vigorous shaking. The culture was transferred to an eppendorf tube and spun in an eppendorf centrifuge at 14,000rpm for two minutes. After discarding the supernatant, the cell pellet was well resuspended in 100µl of cold fresh TES medium and 0.25ml of 100mg/ml lysozyme. The cell suspension was left at room temperature for five minutes before 200µl of 0.2N

NaOH, 1 % sodium dodecyl sulphate (SDS) was added and mixed well. The mixture was incubated on ice for five minutes and then 150µl of cold 3M sodium acetate (pH4.6) was added. After five minutes on ice the mixture was spun in an eppendorf centrifuge for 10 minutes. The supernatant was carefully drawn off and the nucleic acids precipitated with two volumes of ethanol. The DNA pellet was finally washed twice with 70% ethanol, air-dried and resuspended in 50µl TE.

2.3.1.3 Isolation of peripheral blood mononuclear cell (PBMNC) DNA

(modification of Wyman 1980)

Blood samples were collected in 10ml tubes containing EDTA and were allowed to cool to room temperature before being stored at -70°C. For DNA lymphocyte isolation, the frozen blood sample was thawed and transferred to a 50ml centrifuge tube. Cell lysis buffer was added to the tube to 30mls. After mixing, the tube was left on ice for 30 minutes. The cell suspension was spun in the Jouan centrifuge at 3500rpm for 15 minutes at 4°C. The supernatant was aspirated down to 5mls, then cell lysis buffer was added again to 30mls. Centrifugation was repeated. The supernatant was carefully aspirated and 3.25ml Proteinase K buffer, 0.5ml of 10% SDS and 0.1-0.2ml of Proteinase K (10mg/ml) were added and well mixed with the cell pellet. The tube containing the cell suspension was sealed with parafilm, secured on a rotating wheel (10rpm) and incubated overnight at 37°C. DNA extraction was performed twice with phenol and twice with phenol/chloroform. Following ethanol precipitation the DNA pellet was dissolved in 0.1ml of TE.

2.3.1.4 Preparation of bone marrow-derived mononuclear cell DNA

(alternative PBMNC isolation method)

The blood/bone marrow was centrifuged at 2000rpm for 10 minutes. The platelet rich plasma was removed without disturbing the buffy coat. Two volumes of PBS were added to the cells and mixed. A 1/5th volume of Ficoll-Hypaque was carefully layered under the diluted cells and then centrifuged at 1400rpm for 25 minutes. The mononuclear cells were drawn off from the interface with a sterile plastic transfer pipette into a sterile yellow top tube. PBS was added to 10mls and mixed. This was centrifuged at 2000rpm for 10 minutes and the pellet resuspended in PBS, then a second wash repeated. The DNA was then extracted using the method in 2.3.1.3 excluding the additions of lysis buffer.

2.3.1.5 Single stranded M13 DNA

M13 were picked by touching the surface of the plaque with a sterile toothpick and inoculating 1.5ml 2 x TY broth in a 10ml tube. This was shaken at 37°C for 5-8 hours. The cells were transferred to an eppendorf tube and centrifuged for five minutes at 14,000rpm. The supernatant was carefully removed using a Pasteur pipette until the bacterial pellet started to move from the sides of the tube. Centrifugation was repeated and the remaining supernatant carefully removed. To the supernatant 200µl of 2.5M NaCl, 20% polyethylene glycol $(PEG)_{6000}$ was added. After mixing, the tube was incubated at 4°C overnight. The precipitated phage was pelleted by a 15 minute centrifugation in an eppendorf centrifuge. The supernatant was removed and discarded and any residual PEG was carefully wiped away from the inner walls of the centrifuge tube using a tissue. The phage pellet was dissolved in 18µl H_20 and 2µl of 10 x lysis buffer was added. The suspension was incubated at 80°C for

ten minutes then allowed to cool to room temperature. Lysed phage were centrifuged at room temperature for fifteen minutes at 12,000rpm, washed once in 70% ethanol, air-dried and redissolved in 3µl H_20. Phage DNA was stored at -20°C.

2.3.1.6 Purification of DNA

2.3.1.6.1 Phenol, phenol/chloroform extraction of DNA

Solutions of DNA were extracted with phenol/chloroform to remove proteins and other contaminants. Equal volume of phenol (TE saturated, l0mM Tris HCl pH8.0, lmM EDTA) were added to the DNA solution and vortexed vigorously for one minute. After vortexing, the mixture was centrifuged for five minutes at full speed in an eppendorf centrifuge. The upper aqueous phase which contained the DNA was removed leaving a white interface of denatured protein and the lower organic phase. When small quantities of DNA were being handled, the organic-phase was re-extracted with TE and the aqueous phases pooled. For better phase separation and optimal purification of DNA by this method, phenol extraction was sometimes followed by a phenol/chloroform extraction. Here, 0.5 volume of phenol and 0.5 volume of chloroform were added to the DNA solution, vigorously mixed and centrifuged for five minutes. The aqueous phase was removed and added to an equal volume of chloroform: isoamyl alcohol (24:1). The vortex, centrifugation and aqueous phase removal steps were repeated once again. Following either extraction procedure, the DNA was then ethanol precipitated.

2.3.1.6.2 Ethanol precipitation of DNA

The DNA sample was made 300mM with respect to sodium acetate using a 3M stock solution at pH5.2. 2.5 volumes of cold ethanol were added and the tube mixed well. The mixture was incubated at -20°C for one hour or longer. Precipitated DNA was pelleted at 14,000rpm for ten minutes and washed once in 70% ethanol. After drying in air or under vacuum, the DNA was redissolved in H_20 or TE.

2.3.1.7 Preparation of agarose beads containing mammalian DNA/cell line DNA or yeast DNA (refer to 2.6.1).

2.3.1.8 Recovery of DNA from agarose gels

2.3.1.8.1 Prep-a-Gene

The following protocol was obtained from the Biorad Prep-a-Gene handbook, the reagents used in this protocol were provided in the form of a Prep-a-Gene kit. Agarose containing the DNA band was excised from the ethidium bromide (EtBr) stained agarose gel. The wet weight of the agarose gel slice was determined to estimate its volume (1mg = 1ml). Three volumes (to the volume of the gel slice) of binding buffer was added to the gel slice in an eppendorf centrifuge tube and the tube was placed in a 50°C water bath until the agarose had completely melted. Frequent inversion of the tube was required to enable the agarose to melt. 5µl of well mixed silica matrix suspension was added to the solution (5µl for up to 5µg DNA in the gel, an additional 1µl of matrix was added to every additional 1µg of DNA contained in the gel slice) and the solution was left to stand for five minutes at room temperature to allow the DNA to bind to the silica matrix. The bound DNA was pelleted by spinning in an

eppendorf centrifuge at 14,000rpm for twenty seconds. The bound DNA was washed 2x with 10-50 volumes (of the silica matrix volume) of binding buffer and 3x with 10-50 volumes of washing buffer. The DNA was finally eluted from the silica matrix with TE at 50°C for five minutes.

2.3.1.8.2 Electro-elution

(modification of Maniatis 1982)

DNA was recovered from low melting point (LMP) or normal agarose (Pharmacia) gels by electroelution after ethidium bromide (EtBr) staining and visualization with UV illumination. The dialysis tubing used for electroelution was prepared by boiling the tubing for 10 minutes in one litre of 2% sodium bicarbonate, 1mM EDTA. After rinsing thoroughly in distilled water, the dialysis tubing was boiled for 10 minutes in distilled water and allowed to cool and stored at 4°C. The tubing was washed inside and out with distilled water before use. The gel slice containing the DNA to be electroeluted was placed into pretreated dialysis tubing containing 0.5x TBE, 10mM EDTA. DNA was electrophoresed out of the gel slice at 18mA for a duration from 6 hours to overnight in 0.5x TBE depending on the size and amount of DNA to be electroeluted. The current direction was reversed for 2 minutes and the buffer containing the DNA was recovered from the dialysis tubing. DNA was recovered by ethanol precipitation.

2.3.2 Subcloning of human DNA sequences

(Modification of Maniatis 1982)

2.3.2.1 Preparation of plasmid vector DNA and human DNA inserts

500ng of vector DNA (M13mpl8/mpl9, pUCl9, Bluescript phagemid vector SK II) was digested with a restriction endonuclease which cleaves the polylinker, in a total volume of 20µl at the required temperature for one hour. Digestion was tested by running 1µl of digested and undigested vector DNA samples side by side on a minigel which was stained with EtBr and visualised under UV light. Human DNA (cloned in YAC or cosmid, or a PCR product) was digested with the same restriction enzyme that cleaved the vector. The digested DNA sample was checked on a minigel for complete digestion then was extracted once with an equal volume of phenol/chloroform followed by ethanol precipitation. Final DNA concentration of insert DNA was adjusted to 200ng/µl with TE.

2.3.2.2 Dephosphorylation of vector DNA

In order to prevent self ligation of plasmid vector digested with a single restriction enzyme, the 5′ terminal phosphate group was removed with alkaline phosphatase. The vector DNA was digested to completion with an appropriate restriction enzyme in a total volume of 20µl. Then 2µl (1/10 volume) of 10mM EDTA pH 8.0 and 1µl of 2.8U/µl of calf intestinal alkaline phosphatase (CIAP) were added to the digests. The restriction digest reaction was carried out at 37°C for 30 minutes, then 1µl of CIAP was added and incubation was continued for another 30 minutes at 37°C. Then 5µl of 5% SDS was added and the mixture was heated to 65°C for 10 minutes. After extraction with phenol/chloroform, DNA was precipitated with 2 volumes of ethanol. The DNA pellet was rinsed with two changes of 70% ethanol at room

temperature to remove all traces of SDS, desiccated and dissolved in 50µl of TE. To test the efficacy of dephosphorylation, 1µl of dephosphorylated vector was ligated and transformed into *E. coli* strain MV1190. If the 5′ terminal phosphate group was removed, the vector could not recircularize, therefore, only a few colonies would be seen on the agar plate due to the poor efficiency of linear plasmid DNA in transforming *E. coli*.

2.3.2.3 Ligation Reactions

Ligation reactions were carried out with a vector:insert molar ratio of approximately 1:3 to maximise intermolecular ligation rather than intramolecular ligation. Usually, for 100ng of linearized and phosphatased vector, 2µl of 10x ligation buffer, 1-2 units of T4 DNA ligase and insert DNA (200ng) were added and the reaction mixture (in a total volume of 20µl) was incubated at 12-16°C overnight. The efficiency of the ligation reaction was normally checked by re-ligation of the HindIII digested lambda DNA under the same conditions as the sample DNA. The re-ligated and non-re-ligated lambda DNA samples were separated on an agarose minigel. The disappearance of low molecular weight bands and increasing intensity of the large molecular weight bands indicated efficiency of the ligation reaction.

2.3.2.4 Competent cells and transformation

(modification of Chung 1989)

E. coli strain MV1190 cells for M13 were made competent with a method modified from Chung et al., (1989). Stationary phase MV1190 cells from an overnight culture were diluted 1:100 (v/v) into 20ml LB (TY for M13 transformations). The cells were grown at 37°C with constant shaking for 2-2.5 hours. The cells were pelleted by centrifugation in the Jouan

centrifuge at 3,000rpm for ten minutes and then the cell pellet was resuspended in 2mls (1/10th of the original volume) of ice cold fresh TSB. The competent cells were ready for use after leaving on ice for ten minutes. M13 transformations were plated out at this stage. An aliquot of the transformation was added to 100µl of log phase cells, 30µl of 0.1M isopropylthio-D-galactoside (IPTG) 30µl of 2% 5-bromo-4-chloro-3-indolyl D-galactoside (x-gal) and 3 mls of molten H-top agar (45°C). This was mixed and poured onto an 86mm 2 x TY plate. After the top-agar had set, the plate was incubated overnight at 37°C. Recombinant M13 phage were detected as white plaques amongst blue plaques comprising wild type phage. If transformed cells containing plasmids/cosmids were to be selected by appropriate antibiotic resistance, the transformation reaction was diluted with 1ml of LB and then incubated at 37°C for thirty minutes. The cells were then pelleted by gentle centrifugation and resuspended in 100µl of LB. The resuspended cells, 30µl of 2% X-gal and 30µl 0.1M IPTG were spread onto appropriate antibiotic plates and incubated overnight at 37°C. Recombinant plasmids/cosmids were detected as white colonies.

2.3.3 Enzyme digestion, gel electrophoresis and southern blot analysis

2.3.3.1 Restriction endonuclease digestion of DNA

Restriction endonuclease digestion of DNA was carried out using the buffer systems provided by New England Biolabs (see product handbook of Biolabs). Generally, four units of enzyme was added for each microgram of DNA to be digested and the reaction mix was incubated for at least 12 hours for genomic DNA (plasmid, cosmid and phage DNA digests were incubated for 2-4 hours) to ensure complete digestion. To ensure that the enzymic activity was not affected by glycerol, the volume of restriction enzyme(s) did not exceed 1/10th of the final

volume of reaction mix, especially when two or more different enzymes were used simultaneously. Reactions were terminated by the addition of 0.1 volume of 10 x agarose gel loading buffer.

2.3.3.2 Restriction endonuclease digestion of agarose beads

(See 2.6.2).

2.3.3.3 Gel electrophoresis of DNA

2.3.3.3.1 Agarose gel electrophoresis

Electrophoresis of DNA to be used for Southern blot analysis was carried out using agarose (0.8% - 1.2%) dissolved in 0.5 or 1 x TBE and cast on 14cm x 11cm x 0.3cm perspex horizontal casts. Electrophoresis was performed in BRL horizontal tanks containing 0.5-1 x TBE buffer at 15-100mA, until the bromophenol blue had migrated an appropriate distance to ensure that adequate separation of the DNA fragments had taken place. Analytical agarose minigels (for checking digestions etc) were electrophoresed for one hour at 100 volts in a Biorad Mini-sub™ DNA cell. DNA was visualised under UV light after staining the gel in 0.02% ethidium bromide solution for 10-30 minutes.

2.3.3.3.2 Polyacrilamide gel electrophoresis

Polyacrylamide gel mix comprised of 3.5-5% polyacrylamide (acrylamide:bisacrylamide = 19:1) in 1 x TBE with 7M urea. The mixture was filtered through millipore filter paper and added to 0.1% fresh ammonium persulphate and 0.05% TEMED before pouring the gel. The gel was poured and formed between glass plates separated by 0.25mm (for sequencing) or

0.4mm (for microsatellite genotyping). The gel was pre-electrophoresed in 1 x TBE until the gel temperature reached 45-50°C. 3-6µl of denatured sample was loaded per slot and the gel run at 2,000-2,500 volts based on the gel temperature (the best temperature being 50°C) for the appropriate time, depending on the size of the product being separated. After electrophoresis, the gel was transferred to a filter paper, covered with plastic film and dried at 80°C for 2 hours using a 583 gel drier (Bio Rad). The dried gel was exposed to X-Omat XK-1 film (Kodak).

2.3.3.4 Molecular weight markers

EcoRI digested SppI phage or DRIgest were used as molecular weight markers in Southern blot analysis.

2.3.4 ^{32}P radio-isotope labelling of DNA

2.3.4.1 5′ end-labelling of oligonucleotides

Synthetic oligonucleotides were 5′ end labelled using T4 DNA polynucleotide kinase and γ-^{32}P-dATP as described by Chaconas (1980) with the addition of spermidine to a final concentration of 0. 1mM.

2.3.4.2 Primer extension

Labelling of double stranded DNA was performed by primer extension of random oligonucleotides (Feinberg 1983) using the Amersham Multiprime DNA labelling systems kit. In brief, a small quantity of DNA insert (25-50ng) was denatured at 100°C for two minutes and added to a solution containing random hexamers, dATP, dGTP, dTTP, α^{32}P-

dCTP, Klenow fragment of DNA Polymerase I and buffer. The mixture was incubated at 37°C for 1 hour or at room temperature overnight.

2.3.4.3 Probe purification

Unincorporated radionucleotides were removed from labelled probe by running the sample through a Sephadex G-50 column. Two drop fractions were collected, the first peak detected by the mini-monitor B counter being saved since it contained the incorporated labelled probe.

2.3.4.4 Pre-reassociation of repetitive DNA

(Sealy 1985)

^{32}P-labelled DNA thought to contain repetitive DNA sequences was pre-reassociated prior to DNA hybridisation. The labelled DNA was mixed with a 2000 fold excess of sonicated human placental DNA (Sigma) and made 5 x SSC. The sample was denatured in 100°C water bath for 10 minutes, cooled on ice for one minute and then incubated at 65°C for 1-2 hours. The mixture was then added to prewarmed hybridisation mix, and applied to the Southern blot filters.

2.3.5 Southern transfer of DNA to nylon membranes

(Southern 1987, Reed 1985)

Restriction endonuclease digested DNA was separated on agarose gels and transferred to Hybond N+™ (Amersham) nylon membrane using the alkaline transfer method (Reed and Mann, 1985). If the DNA to be transferred was over 1kb in size, an acid nicking step was

included by soaking the gel twice in 0.25M HCl for 15 minutes with gentle shaking. The gel was then immersed in 0.4M NaOH twice for 15 minutes with gentle shaking. The nylon membrane was cut to the size of the gel and placed in the transfer solution (0.6M NaCl, 0.4M NaOH) briefly before transfer. DNA in the agarose gel was transferred to the prepared filter (using the transfer solution) by capillary action for 1-16 hours. The DNA was then washed in 2 x SSC and allowed to dry at room temperature.

2.3.6 Prehybridisation, hybridisation and washing

2.3.6.1 With oligonucleotide probes

Prehybridisation was carried out in a solution consisting of 20% (v/v) 5 x P (1% (w/v) bovine serum albumin; 1% (w/v) polyvinylpyrrolidone Mr 40,000; 1% (w/v) ficoll Mr 40,000; 250mM TrisHCl pH7.4; 0.5% (w/v)pyrophosphate); 1M NaCl; 1% SDS; 10% (w/v) dextran sulphate and 100μg/ml denatured salmon sperm DNA for at least one hour with shaking. Hybridisations were performed in the same solution and incubated overnight at 42°C with between 1-10ng/ml of 5′ end-labelled probe. Filters were washed under conditions determined by the melting temperature of the primer and target sequence. Usually, two washes in 6 x SSC, 0.1% SDS for 5-10 minutes would suffice. If necessary the wash solution could be gradually increased to 65°C. Autoradiography was carried out at room temperature or, for detection of low levels of radioactivity, at -80°C in the presence of tungsten intensifying screens.

2.3.6.2 With oligolabelled probes

Prior to hybridisation, nylon filters were prehybridised at 42°C for 1 hour in a solution consisting of 50% (v/v) deionized formamide, 5 x SSPE, 2% SDS, 5 x Denharts and 100g/ml salmon sperm DNA. Filters were then further prehybridised in the same solution with the addition of 10% (w/v) dextran sulphate for a further one hour. Hybridisations were performed in the same solution and incubated overnight at 42°C with between 1-10ng/ml of oligolabelled probes. The filters were washed twice for 10 minutes in Wash A (2 x SSPE, 1% SDS) at 42°C. They were then washed twice for 10 minutes in wash A at 65°C. Finally the filters were washed twice for 15 minutes in wash B (0.1 x SSPE, 0.1% SDS) at 65°C. Autoradiography was carried out at -80°C in the presence of tungsten intensifying screens.

2.3.7 Pulsed field gel electrophoresis

For the construction of a long range physical map encompassing the long arm break point of the inv(16), pulsed field gel electrophoresis (PFGE) provided the frame work for the separation and analysis of high molecular weight human DNA fragments and cloned human DNA (yeast artificial chromosomes, cosmids). In this project the Pulsaphor Plus 2015 (LKB) with Chef insert and Chef Mapper™ (Biorad) electrophoretic systems were used. In brief, the gel was prepared by casting 150ml of 1% agarose in 0.5 x TBE directly into the gel support tray (15cm x 15cm). For each sample, 50-100µl of agarose beads containing high molecular weight DNA was loaded into the well, using cut-off tips. PFGE was performed in 0.5 x TBE at 14°C at the selected switching interval for the required time. The operation and application of the PFGE systems were according to the manufacturer's instructions.

2.3.7.1 Encapsulation of cells in agarose beads for use with PFGE

(Overhauser 1989)

Due to the large sizes of DNA molecules that can be separated by PFGE, careful preparation of the DNA samples was a critical procedure. To minimise shearing and to preserve the integrity of the large DNA molecules it was necessary to isolate the DNA from intact cells after they had been embedded in agarose. The agarose matrix stabilises and immobilises the DNA molecules after the removal of cell membranes and proteins.

2.3.7.1.1 Preparation of agarose beads containing genomic/cell line DNA

Hybrid cell-line cells were scraped from tissue culture plates (peripheral lymphocytes were collected from blood samples) and gently centrifuged. The cell pellet was adjusted to 2 x 10^7 cells with cold PBS. The cells were washed twice in PBS and finally resuspended in a total volume of 5mls. The cell suspension, 1% low melting point agarose in 1 x PBS and a bottle containing paraffin oil were all equilibrated to 45°C. A beaker containing 100ml ice-cold PBS and a magnetic stir bar was placed in an ice bucket on a stir plate set at medium speed. 5ml of 1% low melting point agarose was added to the warmed cell suspension and mixed. 20ml of prewarmed paraffin oil was then added to the suspension of cells in agarose and the mixture was swirled vigorously for 30 seconds to form a uniform emulsion. The emulsion was quickly poured into the cold PBS and stirred for 2-3 minutes. The mixture was transferred to several 50ml centrifuge tubes and centrifuged in the Jouan centrifuge at 3500rpm for 10 minutes. The paraffin oil layer at the top of the tube was removed leaving a layer of beads just below and also at the bottom of the tube. The beads were dispersed by repeated pipetting with a large bore pipette and the centrifugation repeated. After

centrifugation the excess PBS and any unpelleted beads were removed and the remaining pellets were combined in a single tube. Centrifugation was repeated, the supernatant removed and the inside of the tube was carefully wiped with a tissue to remove all excess paraffin oil. 20ml of 1% SDS, 25mM Na_2EDTA pH8.0 was added to the beads and pipetted repeatedly to break up any clumps. The beads were centrifuged and suspended in 20mls 1% (w/v) sarcosyl, 25mM Na_2EDTA pH8.0, 50µg/ml proteinase K, well mixed and incubated overnight at 50°C. After proteinase K digestion the beads were pelleted, the supernatant discarded and 20ml TE containing 0.1M phenylmethylsulphonyl fluoride (PMSF) was added. The beads were mixed, centrifuged and washed twice in TE before storing in TE. Up to l0ml of beads was recovered.

2.3.7.1.2 Preparation of agarose beads containing yeast DNA

200mls of AHC medium (1 litre of AHC medium consists of 6.7mg of yeast nitrogen base, l0mg of casein hydrolysate, 20mg of adenine and 20mg of glucose) in a one litre flask was inoculated with a single yeast clone and grown at 30°C with constant shaking until stationary phase was reached (2-3 days). The yeast cells were pelleted by centrifugation at 3500rpm in the Jouan centrifuge. The supernatant was removed and the yeast cells were resuspended in l0ml SE (75mM NaCl, 25mM Na_2EDTA pH8.0). After two washes in SE the cells were suspended in 4ml SE. Beads were made using the same procedure as already described except for the use of SE in place of PBS. For digestion of the cells, 0.5ml 2-mercaptoethanol, 5mg zymolase (Sigma) was added and the final volume was adjusted to l0ml with SE. The beads were mixed and incubated at 37°C for two hours. The beads were then pelleted,

resuspended in 20ml 1% (w/v) sarkosyl, 25mM Na_2EDTA pH8.0, 50g/ml proteinase K, and incubated overnight at 50°C to lyse the prepared spheroblasts. The beads were rinsed using the same procedure described for beads containing cell line/genomic DNA.

2.3.7.2 Restriction digestion of agarose beads

For restriction endonuclease digestion of beads, approximately 200µl of bead suspension was placed in an eppendorf centrifuge tube. The tube was filled with 1 x restriction enzyme buffer, the beads thoroughly resuspended and allowed to equilibrate for five minutes before pelleting in the eppendorf centrifuge for two minutes. The buffer was removed and the beads were washed twice more with 1 x buffer. Restriction endonuclease was added at a concentration of approximately 2-5units/g DNA. Incubation was performed at the appropriate temperature for at least four hours (usually overnight). The beads were centrifuged and the supernatant removed before addition of 1:10 volume gel loading buffer. Pelleted beads were reduced to approximately 50g volume by the centrifugation and represented up to 3g DNA/50µl (for genomic DNA).

2.3.7.3 Loading agarose beads into the wells

Agarose beads were dry loaded prior to the gel being submerged into the buffer. The DNA sample was loaded into the well using a cut-off tip, the top of each well was sealed with 2-3 drops of 1% low melting point agarose before being lowered into the buffer.

2.3.7.4 Switching intervals

The most critical parameters of resolving large DNA fragments by PFGE is determining the molecular size range of DNA separation required and the switching interval that will achieve the desired result. When using the Pulsaphor 2015 with Chef insert, a variety of switching intervals were tested to separate standard PFGE molecular weight markers (see section 2.3.7.5). The switch time which gave good resolution at the size range of analysis was then used to separate the DNA digests. The Chef Mapper (Biorad) contains an integrated software system whereby the size parameters of DNA separation are logged in and a computer algorithm automatically selects the appropriate switching ratios to maximise resolution at that scale. The type of equipment used, the size range of DNA separation and switching intervals determined for analysis of DNA in this project is described in the relevant chapters.

2.3.7.5 DNA size markers

A combination of commercially prepared lambda DNA and whole yeast chromosomes were used as molecular weight markers. For lower weight DNA separations Lambda HindIII (Pharmacia) provided DNA markers spanning from 2kb-23kb. For higher molecular weight separations, lambda DNA-PFGE (Pharmacia), a preparation of bacteriophage concatemers provided markers over a range of sizes from 50kb-1000kb in 50kb intervals. In addition, yeast DNA-PFGE (Pharmacia) comprising whole Saccharomyces cerevisiae chromosomes were also used for high level separations as the chromosomes range in size from 200kb to 2000kb.

2.3.8 Polymerase chain reaction (PCR)

All PCR's were performed in a Perkin Elmer-Cetus thermal cycler according to the manufacturers instructions. Incubations were performed in a 20µl final volume comprising of 10µl 2 x PCR mix, 6µl $MgCl_2$ (concentration optimised for each pair of primers), 150ng of each primer template DNA, 1 unit of Taq DNA polymerase and sterile water to 20µl. The solution was mixed well and overlayed with one drop of paraffin oil. PCR's were performed with denaturation, annealing and elongation performed at the designated temperature and time depending on the primer pairs being used and the length of PCR product expected. An appropriate number of cycles were performed and positive and negative controls were always included in each set of reactions.

2.3.8.1 PCR primers

Oligonucleotides for PCR were designed to contain a similar proportion of purine and pyrimidine bases, no long runs of any one base and no repeat sequence DNA. In addition, primer pairs were carefully checked at their 3′ ends to avoid the possibility of primer-dimer formation. The standard length of oligonucleotides used in this study was 25-nt however a range of sizes were used when sequence peculiarities determined the length. All oligonucleotides were synthesized using an Applied Biosystems 391 DNA synthesizer in the Department of Chemical Pathology at the Adelaide Children's Hospital.

2.3.8.2 Oligonucleotide deprotection and cleavage

A manual deprotection apparatus which included the synthesis column containing the sythesized oligonucleotide was assembled. 3mls of ammonium hydroxide was pipetted into a

5ml tube. The ammonium hydroxide was drawn into the column/syringe. The needle was inserted into the rubber stopper and the apparatus was allowed to stand for 30 minutes at room temperature. The needle was removed and the ammonia solution was collected in an eppendorf centrifuge tube. The ammonia treatment was repeated two or three times. The ammonia/oligonucleotide solution was diluted to 3mls by combining all the collected ammonia/oligonucleotide solution and the unused ammonium hydroxide in the 5 ml tube. The solutions were mixed and pipetted into two screw topped eppendorf centrifuge tubes and incubated at 55°C overnight.

2.3.8.2.1 Oligonucleotide purification-n-butanol method

(from Sawadogo 1991).

The cleaved and deprotected oligonucleotide in ammonium hydroxide solution was cooled to room temperature. 700µl of oligonucleotide was then added to 7mls of n-butanol in a centrifuge tube (three or four tubes per oligonucleotide were required). The solution was vortexed for 15 seconds, then centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 700µl of water. An additional 7mls of n-butanol was added, then vortexed, centrifuged and supernatant discarded as before. The pellet was dried under vacuum and resuspended in 100 µl of water. The DNA was quantitated and the concentration of oligonucleotide was adjusted to 1mg/ml.

2.3.9 Yeast artificial chromosome (YAC) cloning system

The YAC vector system is suitable for cloning of very large DNA fragments up to several hundred kilobase pairs in size (Burke 1987). This system is particularly appropriate in

physical mapping studies and accelerates the process of chromosome walking and gene cloning.

2.3.9.1 YAC vectors

The YAC vector was constructed from both yeast DNA sequence and plasmid pBR322 derived DNA, and incorporates all necessary functions into a single plasmid that can replicate in Escherichia coli (Burke 1987). All the sequences necessary for an artificial chromosome are carried by the vector. The centromere (CEN 4) autonomous replicating sequence (ARS 1), telomeres (TEL), selectable markers (TRP 1, URA 3) and SUP 4 gene are derived from yeast DNA, whilst ampicillin resistance gene (Amp) and origin of DNA replication (ORI) are from plasmid pBR322. The ARS 1, CEN 4 and TEL sequences confer replication and mitotic/meiotic stability on the YAC during propagation in yeast. Therefore, a YAC can replicate in the same manner as its host's chromosomes. The selectable marker URA 3 is for positive selection of transformants in URA 3 hosts. The Amp gene and ORI are essential for growth and amplification of the YAC vector in E.coli. In each pYAC vector the cloning site is different, such as SmaI in pYAC 2, SnaBI in pYAC 3, EcoRI in pYAC 4 and Not I in pYAC 5, although all of the cloning sites are in SUP 4 gene sequences. When exogenous DNA is cloned into the cloning site, SUP 4, an ochre-suppressing allele of a tyrosine transfer RNA gene, is interrupted and produces red colonies in place of white ones. Since pYAC contains the Amp gene, *E. coli* cells containing any of the pYAC vectors can grow in LB medium plus Amp.

2.3.9.2 YAC library

The YAC clones analysed in this project were isolated from a YAC library constructed by Dr D LePaslier and colleagues at the Centre d'Etude du Polymorphisme Humain (CEPH), Paris, France. A complete human YAC library was produced as it was intended to serve as a source for high density physical mapping for the entire human genome. The library was constructed by ligation of partial EcoRI digested total human DNA with the pYAC 4 vector. The vector had been digested with EcoRI (cloning site) and BamHI. The details of library construction, are detailed below in 2.3.9.3 and have been published elsewhere (Burke 1987). The other YAC library source was that established at the Los Alamos laboratories, by similar methods as above. This was used in the latter part of this project for the mapping of the genes *MRP* and *ARA* with respect to *MYH11*.

2.3.9.3 YAC cloning strategy

The strategies for cloning large human DNA fragments have been reported (Abidi 1990, Burke 1987). In general, the pYAC 2 vector is prepared by double digestion with BamHI and SmaI (cloning site). Three fragments are generated: the left arm (including the centromere), the right arm, and a discard region that separates the two TEL sequences in the circular plasmid. The two arms are then treated with alkaline phosphatase to prevent re-ligation. Large human DNA inserts, obtained by partial digestion of high molecular weight human DNA with a restriction enzyme that leaves SmaI compatible ends (i.e. blunt ends) are ligated to the YAC vector arms. The ligation products are then transformed into yeast spheroblasts. The transformants containing an extra linear form YAC are selected for complementation of a trpI marker, which ensures that the YAC contains both arms of the vector. Finally, they are

tested for loss of expression of SUP4 gene, which is interrupted by insertion of exogenous DNA at the SmaI cloning site (Burke 1987). The final structure of a YAC, is that of a human DNA insert located between right and left YAC vector arms.

2.3.10 Cosmid vectors

Cosmid vectors were originally designed to clone and propagate large segments of genomic DNA. In their simplest form, cosmid vectors are modified plasmids that carry the DNA sequences (cos sequences) required for packaging DNA into bacteriophage lambda particles. Because cosmids carry an origin of replication and a drug resistance marker, cosmid vectors can be introduced into *E. coli* by standard transformation procedures and propagated as plasmids. Cosmid vectors have been constructed to contain a large variety of structural elements designed to improve sequence representation, to simplify or expedite the structural or functional analysis of cloned DNA, or to simplify the construction of high quality representative libraries.

2.3.10.1 Scos vectors

Scos vectors are a family of vectors that have been designed specifically for the mapping and functional analysis of human chromosomes. The construction of these vectors, described elsewhere (Evans 1989), included the presence of two cos sites so that packaging could be carried out with high efficiency and without requiring size selection of the insert DNA. The vectors also include the presence of T7 and T3 bacteriophage promoters for the synthesis of "walking" probes. Unique restriction sites were incorporated for the removal of the insert from vector, for example; Not I sites for Scos1, Not I and Sac II sites for Scos2 and Sfi I sites

for Scos4. To aid in restriction mapping, the vectors contained selectable genes for gene transfer in eukaryotic cells (i.e. Amp and SV2 Neo). A plasmid origin of replication (ori) was also included for giving high yields of cosmid DNA when preparing templates. In this project Scos1 cosmid vector was used.

2.3.10.2 Cosmid cloning strategy

The cosmid cloning strategy has been described in detail elsewhere (Hohn and Collins, 1980, Chia 1982). In brief, segments of foreign DNA approximately 35-45 kb in length are isolated and ligated to linearized cosmid vector DNA. Two cos sites, arranged in the same orientation flank the foreign DNA. Also located within this region of DNA is an entire complement of plasmid genes. These complexes are then used as substitutes in an in vitro packaging reaction whereby the cos sites are cleaved by the ter function of the bacteriophage lambda gene. A protein and the DNA between the two cos sites is packaged into mature bacteriophage lambda particles. Bacteriophage lambda heads will accommodate up to 52kb of DNA (Williams and Blattner, 1979) therefore with conventional cosmid vectors accounting for up to 5kb of DNA, recombinant cosmids containing up to 47kb of foreign DNA can be constructed. The smallest piece of DNA that can be packaged into bacteriophage lambda particles is approximately 38kb (Williams and Blattner, 1979) so the minimum size of DNA that can be cloned is approximately 33kb. During infection of *E. coli* by the bacteriophage particles, the linear recombinant DNA is injected into the cell, and via the cohesive ends of the cos sites the DNA is circularized. The resulting circular molecule contains a complete copy of the cosmid vector and replicates as a plasmid conferring drug resistance upon its bacterial host.

Therefore bacteria carrying recombinant cosmids can be selected using media containing the appropriate antibiotic.

2.3.10.3 Construction of a chromosome 16 specific ordered cosmid library

The chromosome 16 specific cosmid library was constructed at the Los Alamos National Laboratory, New Mexico and has been described (Stallings 1990). In general, human chromosomes 16 were isolated from a somatic cell hybrid CY18. A single chromosome 16 was the only human chromosome present in this hybrid. After partial digestion with Sau3A and dephosphorylation with calf intestinal alkaline phosphatase, the chromosomal DNA was ligated to the cloning arms from the cosmid vector Scos1. In vitro packaging and infection of *E. coli* yielded 1.75 x 105 independent recombinants, giving a 67 fold statistical representation.

2.3.10.3.1 Construction and screening of high density cosmid grids

(performed at Los Alamos National Laboratories, New Mexico).

A Beckman Biomek 1000 was used to stamp bacterial colonies onto Biodyne nylon hybridisation membranes (1536 clones/membrane) as already described (Longmire 1991). Membranes were hybridised overnight in 6x SSC, 10mM EDTA pH 8.0, 10X Denhardts, 1% SDS, 0.1mg/ml denatured sonicated salmon sperm DNA, at 65°C. Following hybridisation membranes were washed in 2 x SSC, 0.1% SDS at room temperature (quick rinse); once in 2x SSC, 0.1% SDS at room temperature for 15 minutes; and twice in 0.1% SDS at 50°C for 30 minutes. Probes were labelled with ^{32}P to a specific activity of 108cpm/μg by primer

extension labelling (2.3.4.2) and pre-reassociated (2.3.4.4) prior to hybridisation of the probe to the filters.

2.3.10.3.2 Repetitive sequence fingerprinting and assembly of contigs

(performed at Los Alamos National Laboratories).

An approach has been developed for the identification of overlapping cosmid clones by exploiting the high density of repetitive sequences in complex genomes as described by Stallings (1990). By coupling restriction digestion mapping with oligomer probes targeting abundant interspersed repetitive sequences such as Alu (Jelinek 1982, Tagle 1992), Ll (Scott 1987) and (GT)n (Rich 1984, Weber 1989), a "fingerprint" is obtained. The initial analysis of fingerprint data are the pairwise comparison of the fingerprint between cosmid clones. Clones that overlap will share restriction fragments of similar size with similar repetitive sequences. A probability of overlap is assigned to each pair of clones based on the number of shared restriction fragments with the same repetitive sequences. The analysis of the treatment of fingerprint data and the algorithm used to detect pairwise overlap has been described (Balding 1991). Once overlapping pairs have been identified, the clones are ordered and assembled into contigs based upon the information in overlapping pairs. For contig construction a computer programme was used based on a genetic algorithm - genetic contig assembly algorithm, GCAA (Cinkosky 1991). GCAA represents possible maps as strings of numbers encoding the lengths and positions of the clones. The quality of any possible map is measured by a fitness function that takes into account the most likely overlaps, overlap extents and clone lengths that the map is intended to fill. By the repetitive sequence fingerprinting of approximately 4000 cosmid clones obtained from the chromosome 16

specific library, a cosmid contig map was developed (Stallings 1992a). The clones were organised into 576 contigs and 1171 cosmid clones not contained within a contig. Similar strategies were used in the assembly of the cosmid contig generated from the data obtained in chapter 3, however many orders of magnitude less complicated than the contig assembly generated at the Los Alamos National Laboratories.

2.3.10.4 Isolation and hybridisation of cosmid end-probes

Cosmid DNA was digested with a range of restriction enzymes including PstI, HindIII and XmnI according to the manufacturer's instructions. The digests were electrophoresed and nylon filters were prepared by Southern blot transfer (2.3.5). DNA fragments representing the terminal sequences of the genomic insert were identified by the successive hybridisation of T7 and T3 bacteriophage promoter sequences to the filters. These sequences flank the Scos1 Not I cloning site. T7 and T3 oligomers were labelled by 5'-end labelling (2.3.4.1). Prehybridisation, hybridisation and washing of the filters have been described (2.3.6). Preparative gels were made and the DNA fragments that hybridised to the T7 and T3 oligomers were excised and purified by the Prep-a-Gene method (2.3.1.8.1). The length of genomic DNA represented in either T7 or T3 generated end-probes was determined by subtracting the length of vector DNA present from the total length of the isolated DNA fragment. For each restriction enzyme used to generate an end-probe, the amount of vector DNA present was calculated by establishing the distance from the cloning site to the first restriction enzyme site in either the T7 or T3 end of the vector. For example, the T7 and T3 vector lengths have been calculated for PstI, and XmnI restriction enzymes and are (2kb/0.7kb) and (0.27kb/0.4kb) respectively.

2.3.10.5 Isolation of repeat free sequences from restricted cosmid DNA

Cosmid DNA frequently contains high level repeat sequences which bind non-specifically to other repeat sequences throughout the genome. This leads to poor quality probe hybridisation and spurious contig overlaps in the construction of cosmid contigs. A simple method for circumventing this problem and to obtain more unique probes to be used for the purpose of mapping and alignment of cosmids, involved the following. The cosmid DNA was cut with a number of 4 and 6 base pair cutter restriction endonucleases: eg. AvaI, PstI, HaeIII, XbaI, SacI, BglII, HincII, HindIII, EcoRI as per 2.3.3.1. Half of the restricted DNA was then run out on a 1% agarose gel to separate the restriction fragments and then Southern transferred. The resultant membrane was then hybridised with labelled total human DNA, washed and exposed to radiographic film for 12 - 24 hours. The resulting autoradiograph was then compared with the restriction bands on the photograph of the gel and those bands not hybridising to the total human DNA were those restriction fragments which contained no or few repeat sequences. These bands were then excised from a second agarose gel and subsequently used for probing either other restricted cosmid, YAC or total human DNA.

CLONING THE Q ARM BREAKPOINT OF INV(16)

Chapter 3

3.1 Introduction

Increasingly, cytogenetic analysis of leukaemias has assumed an important role. Initially this was in identifying the clonality of the disorder but subsequently in the recognition of pathogenetic implications of the chromosomal rearrangement. As the chromosomal breakpoints are further analysed at the molecular level, aetiological and mechanistic understanding of the events causing and propagating the leukaemia is increased. The inv(16) abnormality has already been identified as being predominantly linked with myelomonocytic differentiation of bone marrow precursors, linked with abnormal eosinophil differentiation within the bone marrow (LeBeau 1983) and with an improved survival as compared with other leukaemic subtypes (Larson 1986). This has been discussed in detail in the introductory chapter of this thesis. The expectation in attempting to clone the breakpoints of the inv(16) leukaemia is that the genes involved will have implications for abnormal myeloid differentiation and transformation of a stem cell into a malignant phenotype.

Considering examples of previously cloned breakpoints, the types of genes involved in leukaemogenesis include transcriptional regulating factors (*BCL3* [t(14;19)] (Ohno 1990), *AML1* [t(8;21)]) (Miyoshi 1991); DNA binding proteins (*MLL*[11q23] (Thirman 1993), *PML & RARA*[t(15;17)] (Kakizuka 1991); homeobox genes (*PBX* [t(1;19)] (Rowley 1990), tyrosine kinases (*ABL* [t(9;22)]) (Groffen 1984) as well as a range of other oncogenic factors. This provides a list of potential gene families which become oncogenic when altered by translocation, inversion or mutation. However, the approach to the genetic mapping of a specific breakpoint which passes through a single gene, is quite different to the ascertainment

of candidate tumour suppressor genes where there is loss of heterozygosity of a region containing a number of genes. Those candidate genes belonging to the deleted region most likely to be involved in oncogenesis are then subjected to mutational analysis. Oncogenes and tumour suppressor genes involved in translocations may be inactivated or altered in their expression through chromosomal rearrangement. These rearrangements may be primary or secondary events in the progression of the malignant disease (Knudson 1987, Adams 1992) and can be detected by comparison of DNA extracted from the nuclei of malignant cells with DNA extracted from normal tissue or chromosomal abnormalities seen in metaphase spreads from the malignant cells. Once the molecular rearrangement at the site of a breakpoint is known and the gene(s) have been identified, more sensitive detection methods can be employed to search for the presence of the molecular rearrangement at times of diagnosis, remission and relapse of the disease.

This chapter describes the work aimed at cloning the long arm breakpoint of the inv(16) and sequencing and characterising the gene(s) involved. The long arm breakpoint was chosen as a starting point for the positional cloning in view of the fact that a number of reports of acute myeloid leukaemia have described 16q22 involved in translocations with other autosomes (Yip 1991, Murakami 1991) and that the del(16q22) chromosomal abnormality results in a similar leukaemic subtype without involving a short arm gene. This presumes that the breakpoint for the del(16q22) involves the same gene as the inv(16)(p13q22.1). Also, the long arm breakpoint was preferred due to the presence of a number of DNA repeat sequences being more prevalent in the short arm breakpoint region and due to a high density of anonymous probes present in the region of the long arm breakpoint. The expectations were

that the more important gene in terms of leukaemogenesis was to be found at the long arm breakpoint but that in cloning this breakpoint, the short arm gene would be subsequently identified. Further aims were to isolate both normal and abnormal cDNA species from leukaemic and normal bone marrow cells to demonstrate one or two chimeric gene products secondary to the inversion and to develop an RT-PCR screening test for the presence of the molecular disruption of these genes.

Unfortunately before this work could be successfully completed, the breakpoints were cloned by a team of scientists in the United States of America (Liu 1993). The chapter presents the experiments and results to the stage reached when the cloning of the inv(16) was reported. At this time the direction of the thesis changed. For the sake of continuity, the relevance of the *CBFB* gene and the related gene *AML1,* involved in the t(8;21) breakpoint, is fully discussed in the conclusion and discussion section of this chapter.

3.2 Work prior to commencement of thesis:

3.2.1 Isolation of a somatic cell hybrid containing the inverted chromosome 16

A mouse/human somatic cell hybrid was created by fusing A9 cells with bone marrow cells (Callen 1986) known to have the inv(16) associated with myelomonocytic leukaemia. Screening of these hybrids allowed the isolation of the line CY10, containing a leukaemia derived inversion chromosome 16 with an intact *APRT* gene (Fratini 1986) in particular excluding the normal human chromosome 16. This hybrid, formed part of an extensive mouse/human somatic cell hybrid panel of chromosome 16 (Callen 1992). Using metaphase chromosome spreads from this hybrid and FISH based mapping of anonymous DNA probes

(Hyland 1989, Callen 1988), the long arm breakpoint of the inv(16) was found to map to 16q22.1, to the region between the hybrid breakpoints CY130D and CY4 (Figure 3.1).

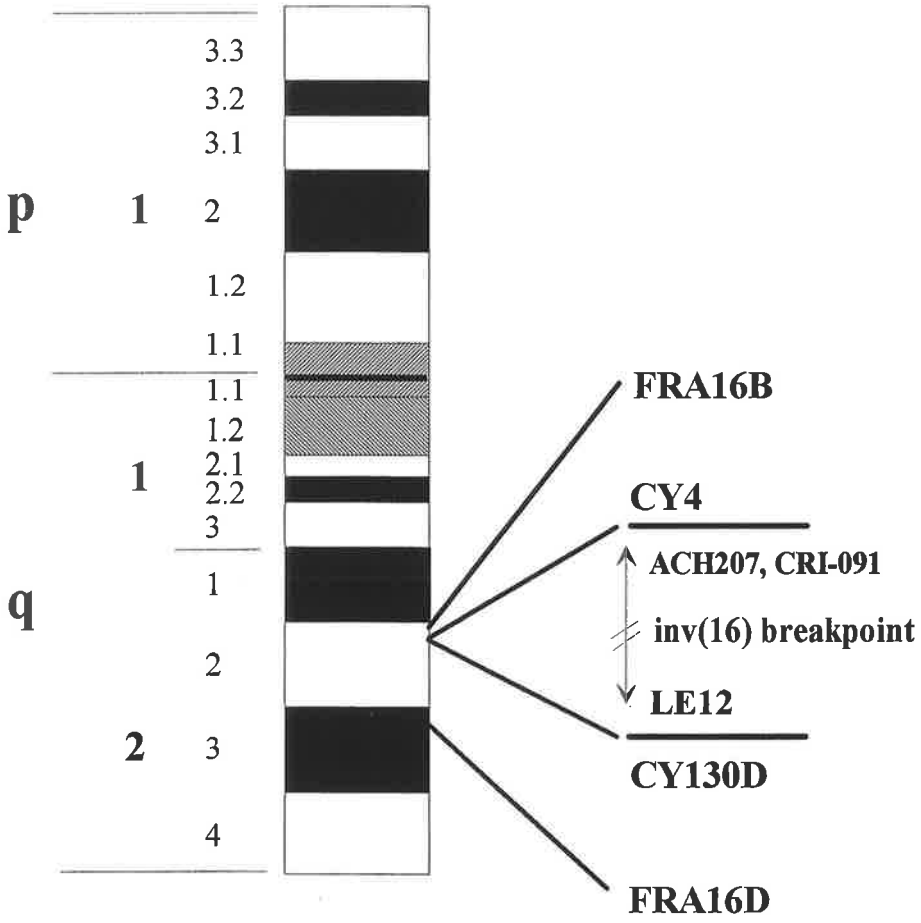
3.2.2 Finding flanking genetic markers by FISH

Anonymous DNA probes were selected from this hybrid interval and analysed by FISH for their position with respect to the long arm breakpoint. The intention was to isolate probes flanking the long arm breakpoint to allow the positional cloning of this breakpoint. Centromeric and telomeric probes were identified such that the long arm inversion breakpoint was positioned distal to the probes ACH207(*D16S4*)[4.7kb HindIII fragment cloned into pBR322] and CRI-091(*D16S46*) [35-40kb MboI fragment cloned into c2RB] and proximal to LE12(*D16S91*)[1.2kb EcoRI fragment cloned into pUC13] (figure 3.1). A fourth probe, CRI-02(*D16S38*) [35-40kb MboI fragment cloned into c2RB], was also thought to be telomeric to the breakpoint however, this result was less certain due to cross hybridisation with DNA repeat sequences proximal to the breakpoint. In addition, pulsed field gel electrophoresis of MLU1 digested human genomic DNA established that ACH207 and LE12 were linked by the same 460kb restriction fragment (Naras Lapsys). This made it likely that these two probes were the closest flanking probes to the long arm breakpoint. The remaining two probes, CRI-091 and CRI-02, were unable to be linked by pulsed field analysis.

Figure 3.1 Map of chromosome 16 adjacent the inv(16) q arm breakpoint

An idiogram of the metaphase appearances of chromosome 16 is shown. The interval between CY130(D) - CY4, on the long arm of chromosome 16 at 16q22.1, is marked. Regional fragile sites for the long arm of chromosome 16 (FRA16B and FRA16D) are highlighted. The flanking markers identified by FISH are shown in the insert and their positions with regard to the long arm breakpoint of the inv(16) are demonstrated.

Chromosome 16



3.3 Materials and methods specific to this chapter

3.3.1 DNA sequencing

3.3.1.1 Sequence gels

Sequence gels were formed between 52cm x 21cm glass plates separated by 0.25-0.4mm wedge spacers. Gel mixes were comprised of 5% polyacrylamide (20 acrylamide:1 bisacrylamide) in 1 x TBE with 7M urea. The mixture was filtered through Millipore filter paper discs and degassed in a vacuum before the addition of 0.1% fresh ammonium persulphate and 0.05% TEMED (N, N, Nl, Nl tetramethylethylenediamine). The gel was poured and wells were formed between the teeth of a 0.25mm sharks tooth comb inserted into the top surface of the gel. The gel was pre-electrophoresed in 1 x TBE for 30 minutes prior to loading so as to warm the apparatus. 3µl of denatured sample (see 3.3.1.5) was loaded per slot and the gel run at 2000 volts for the appropriate time. Up to 500 bp of sequence could be read from wedge gels. After electrophoresis the gel was transferred to a piece of pre-cut filter paper, covered with plastic film and dried at 80°C for 3-5 hours under vacuum on a 583 Gel Drier (Biorad). The dried gel was exposed to X-ray film overnight.

3.3.1.2 DNA sequencing by chain termination

(Sanger 1977)

DNA sequencing by the chain termination method is possible for both single stranded (M13) DNA and double stranded (plasmid) DNA. For single stranded M13 sequencing, the DNA to be sequenced was subcloned into bacteriophage M13 and template DNA was prepared as described in section 2.3.1.4. When sequencing plasmid DNA subclones it was first necessary to denature the double stranded template.

3.3.1.3 Denaturation of double stranded DNA template

(Current Protocols in Molecular Biology 1993)

To an eppendorf centrifuge tube 10µl of template (1-2µg of plasmid DNA containing insert) was denatured by the addition of 2µl 2M NaOH/2mM EDTA. The solution was briefly vortexed and incubated at room temperature for 10 minutes. The solution was neutralised by the addition of 3µl sodium acetate (pH4.5) and 7µl H20. The DNA was precipitated by the addition of 60µl ethanol and incubated on ice for 30 minutes. The pellet was washed with 70% ethanol, vacuum dried and resuspended in 10µl of H_20.

3.3.1.4 Annealing.

10µl of template DNA (M13 template or denatured plasmid DNA) was added to 2µl of 5 x reaction buffer (200mM Tris-HCl, pH7.5, 100mM $MgCl_2$, 250mM NaCl) and 1µl of primer (M13 used 2µl sequencing primer, Applied Biosystems, for pUC sequencing pUC-F and pUC-R primers were used). The mixture was heated to 70°C for two minutes and then allowed to cool to <30°C by placing the tube into 70°C water and allowing the water to gradually cool.

3.3.1.5 Labelling and chain termination

The primer annealed DNA template (13µl total volume) was added to 2µl of labelling mix (1.5M dGTP, 1.5M dATP, 1.5M dTTP), 0.5µl α^{32}P-dCTP and 24µl Sequenase (USB). The solution was mixed and polymerisation allowed to occur at 45°C for five minutes in a water bath. Termination was initiated by the addition of 4µl of the polymerisation reaction to four separate tubes, each containing 4µl of either C, G, T, or A termination mix (each mix

contained either 80μM dGTP, dCTP, dTTP or dATP and 50mM NaCl. In addition each appropriate mix contained either 8μM of ddGTP, ddCTP, ddTTP or ddATP). The samples were well mixed, incubated at 70°C and cooled to room temperature. 4μl of formamide loading buffer was added to each tube, heat denatured at 95°C for five minutes and loaded onto a 5 % sequencing gel.

3.3.2 CEPH YAC library

3.3.2.1 PCR based screening of the CEPH YAC library

The systematic screening of YAC libraries by use of the PCR have been described (Green 1991). The general approach for screening the human YAC library is outlined below. The YAC library screened was one of the original libraries produced, with YAC insert sizes ranging from approximately 200kb to 500kb, as compared with the sizes of the megaYACs currently available (in excess of 2Mb in size).

Individual YAC clones were grown in arrays of 384 colonies per nylon filter (in 96 well microtitre plates). The yeast cells from each filter were pooled and the DNA was purified, yielding single filter pools of DNA. Equal aliquots from single filter pools were mixed together in groups of four to yield multi-filter pools. A total of 113 multi-filter pools were provided to our laboratory, with the single filter pools and the filters containing the human YAC clones remaining at CEPH, France. The multi-filter pools of DNA were then analysed individually for the presence of a specific human DNA segment using PCR. Approximately 7ng of each multi-filter pooled DNA was then added to a 20μl final volume PCR, combining

150ng/µl of each oligonucleotide primer designed for a specific human DNA segment, optimised Mg^{++}, and 0.05units/l Amplitaq. The PCR cycling conditions were: 94°C denaturation for 1 minute, 55°C annealing for 1.5 minutes and 72°C extension for 1.5 minutes, over 25 cycles. A final 72°C extension for 10 minutes was performed when the 25 cycles were completed. Following amplification, the reactions were then separated on an agarose gel, the gel was then ethidium bromide stained and PCR products visualised under UV light. A Southern blot filter of the products was prepared and a radiolabelled PCR product from a PCR reaction using total human genomic DNA as a template and the PCR primers used in the screening, was used as a probe to confirm any positive multifilter pool PCR signals. If DNA from a multi-filter pool was found to generate the appropriate PCR product, then each constituent single filter pool was analysed individually by the same PCR assay. Upon generation of the appropriate PCR product with a single filter pool the location of the positive clone within the 96 well array was established by colony hybridisation using the radiolabelled PCR product as a probe. The single filter pool PCR assay and colony hybridisation was performed at CEPH, Paris, and positive YAC clones in stabs were returned back to our laboratory.

3.3.2.2 End probes for YAC mapping (YAC-L, YAC-R, pUC19).

The YAC vectors were designed so that both end probes were easily identified using the plasmid pBR 322. The end probes had no cross hybridisation with either yeast or human genomic sequences. When constructing the YAC vector, yeast DNA SUP 4 (containing the cloning site) was ligated into the BamHI site of pBR322 DNA (position 375). The pBR322 DNA sequences from the BamHI site (Position 375) to the PvuII site (position 2066)

containing the ampicillin resistance gene (Amp) and an origin of replication site (ORI) form part of the centromere arm (left arm) of the YAC vector, the rest of the pBR322 sequences form part of the no centromere arm (right arm). Therefore, appropriate pBR322 DNA sequences in the left arm or the right arm of the YAC vector can be used as an end probe to detect the corresponding end of a human DNA insert in a YAC. To construct YAC-left (YAC-L) and YAC-right (YAC-R) probes, pBR322 DNA was digested with three restriction enzymes (BamHI, PstI and NruI) to generate three DNA fragments. YAC-L, a 1kb Bam HI/PstI fragment detected the left arm of the YAC; while YAC-R, a 600 BamHI/Nru I fragment detected the right arm. Alternatively, to detect YAC-L, plasmid vector pUC19 contains a 2295bp PvuII/EcoRI fragment of pBR322 (from position 2066 to 4361 containing Amp and ORI), so that it can be used as an end probe instead of YAC-L to identify the centromere arm of the YAC.

3.3.2.3 YAC mapping strategy

In this project the restriction mapping of human insert DNA fragments in YACs was performed primarily to determine whether the YACs 26A8, 12F4 and 50C2 overlapped one another and contained the flanking markers LE12 and ACH207. Since the human DNA fragment in a YAC is usually several hundred kilobases in size, restriction enzymes that can generate large DNA fragments are chosen for long range mapping. In general, agarose embedded high molecular weight YAC DNA (refer 2.3.7.1.2 for methods) was digested to completion with various restriction enzymes. The restriction fragments were separated by PFGE, Southern blotted onto nylon membranes, and successively hybridised with end probes (YAC-R, YAC-L or pUC 19), as well as human DNA probes, that had been labelled by the

primer extension method (2.3.4.2). Standard prehybridisation, hybridisation and washing methods of the filters were performed (2.3.6). The size of a restriction fragment detected by one end probe indicated the distance from the restriction site to the corresponding telomere, so the restriction map could be built up from both ends. Complete digestion with the restriction enzymes can generate a complete map only for the enzymes which have one cleavage site in the human DNA insert. To generate a complete map of all restriction sites, partial digestion and double digestion of the restriction enzymes would have to be performed. However, for the construction of a restriction map to determine YAC overlap, complete restriction mapping of all restriction enzyme sites was not required. The probes internal to the human insert of the YAC DNA, ACH207 and LE12 were also hybridised to the YAC DNA, to determine restriction fragments positive for these probes. Where a restriction fragment for an internal probe coincided with a restriction fragment for an end probe, the internal probe could be positioned with respect to the end of the YAC positive for that internal probe and so a detailed restriction map could be generated.

3.3.2.4 PFGE of restricted YAC DNA

PFGE was performed using the LKB Pulsaphor Plus 2015 apparatus with CHEF inserts by the methods described (2.3.7). High molecular weight DNA fragments were separated in two ranges of resolution.

(i) high resolution PFGE. DNA fragments in the size range of 50kb-900kb were resolved. The switching interval was a 70 second pulse for 24 hours at 150 mA.

(ii) low resolution PFGE. DNA fragments in the size range 200kb-1600kb were resolved. Low resolution PFGE was performed using ramped switching intervals of 200 sec for 12

hours, 150 sec for 12 hours and 100 sec for 12 hours, at 150 mA. Lambda and yeast PFGE size markers (2.3.7.5) were used. Any modifications to the switching intervals are detailed where appropriate.

3.3.3 Construction of a Cosmid Library from YAC DNA

3.3.3.1 Preparation of YAC DNA

YAC DNA was prepared by encapsulation in agarose beads, as per 2.3.7.1.2. and digested with Sau3A restriction enzyme in a time restricted manner so as to result in partial digestion of the YAC DNA with the DNA fragments remaining in the size range of just greater than 23kb. After a number of trial digests using 50µl of YAC beads digested with 0.125-4units of Sau3A enzyme it was determined according to the resultant size of the DNA restriction fragments that 0.125units of enzyme per 500µl of beads digested for 30 minutes at 37°C was optimal. YAC 26A8 beads were then digested using 50µl of 10x Sau3A buffer, 5µl of 0.5M spermidine, 5µl Bovine Serum Albumin at 1mg/ml stock solution and 0.125units of Sau3A enzyme. The digested DNA was then dephosphorylated using calf intestinal alkaline phosphatase (CIAP): 500µl of digested YAC DNA in beads, 50µl CIAP 10x buffer, 20µl of CIAP, incubated at 37°C for 60 minutes. 100µl of 0.1M EDTA pH8 was added to terminate the reaction which was then heated to 65°C for 10 minutes, to inactivate the CIAP. The partially restricted DNA fragments were separated on an agarose gel to determine the size of the restriction fragments. When the correct size was achieved (ie. sized above the 23kb band of the DRIgest DNA size marker) the DNA was then dephosphorylated in a standard procedure (refer 2.3.2.2) with 500µl of YAC beads, 50µl of CIAP buffer and 20µl of phosphatase enzyme and incubated for 60 minutes. This was followed by the addition of

100µl of 0.1M EDTA pH8, to inactivate the enzyme. A 0.4% 0.5x TBE low melting point agarose preparative gel containing the partially digested DNA, was run overnight at 18mA so that the specifically sized DNA could be excised from the gel in a large band of agarose and electroeluted from the agarose by dialysing against a constant current overnight (2.3.1.8.2). The DNA was then precipitated from the dialysate following three volume reductions using n-Butanol and then precipitating in an equal volume of isopropanol. The pellet was then dried and resuspended in 50µl of TE and the concentration checked by spectroscopy.

This method was chosen after a number of different methods were trialed including the use of β-agarase and phenol-chloroform extraction of the DNA however in each case, the method resulted in the loss of a considerable amount of DNA and also the shearing of DNA into smaller fragments unsuitable for packaging into cosmids. The method used was time consuming however reliably resulted in the correct size range of DNA with sufficient DNA at the end of the procedure to ligate to the cosmid DNA for library construction.

3.3.3.2 Preparation of Cosmid DNA

The cosmid sCos1 was selected as the vector to be used for the construction of the cosmid library due to its multiple cos sites allowing for simple and efficient cloning (Dilella 1987). The cosmid DNA was digested to completion with XbaI restriction endonuclease. The digest was then checked on a 1% agarose gel stained with ethidium bromide and if satisfactory (a single linear 7.6kb band visualised) the cosmid DNA was extracted using phenol and chloroform as per 2.3.2.6.1., precipitated with 100% alcohol and resuspended in 10µl of TE. The vector DNA was then dephosphorylated as per the methods outlined in 2.3.2.2) and once

again phenol chloroform extracted. The DNA was lyophilised and resuspended in TE to a final concentration of 1μg/μl and digested to completion with BamHI restriction endonuclease (the presence of 1.1kb and 6.5kb bands visible on a 1% agarose gel confirms this). The DNA was again extracted with phenol and chloroform and precipitated with ethanol and resuspended at 1μg/μl in TE. Large scale ligation reactions were then used to ligate the phosphatased, partially Sau3A digested YAC DNA with the prepared cosmid DNA: 1μg of YAC DNA, with 1μg of cosmid DNA, 5μl of 10x ligation buffer, 5μl of 10mM ATP in a 50μl reaction with 1-2 units of T4 ligase. This was incubated at 12-16°C overnight and then used for packaging the following day.

3.3.3.3 Preparation of bacterial host

The host bacteria was the strain LE392. An aliquot from a glycerol stock was streaked out onto LB-broth plates and incubated overnight at 37°C. 50ml of LB media with 10mM $MgSO_4$ and 0.2% Maltose was inoculated with a single colony of the plated LE392 bacteria, and incubated at 30°C in a shaking incubator overnight. The bacteria were pelleted at 2000 rpm for 10 minutes at 4°C. The cells were resuspended in 12.5ml of sterile 10mM $MgSO_4$, then diluted to an optical density of 0.5 with sterile 10mM $MgSO_4$.

3.3.3.4 Packaging Protocol

The packaging kit used for this procedure was the GIGAPACK® II GOLD, Stratagene. The terms used ie. Sonic extract and Freeze/thaw extract are those used by the manufacturer, the details of these extracts has not been provided by the manufacturer.

An appropriate number of sets of packaging extracts were removed from the -70°C freezer and placed on dry ice. Sonic extract was thawed on ice. The Freeze/Thaw extract was thawed and 2-3µl of prepared ligated cosmid-YAC DNA (as per 3.3.3.2) was immediately added and placed on ice. 15µl of the sonic extract was added to the Freeze/thaw extract containing DNA and mixed well. This was then quickly centrifuged in a microcentrifuge for 3-5 seconds and incubated at room temperature for 2 hours (22°C). 500µl of phage dilution buffer (SM buffer) was then added. 20µl of chloroform added to the eppendorf microcentrifuge and tube given a 3-5 second spin in the microcentrifuge to sediment the debris. The supernatant then contained the partially digested YAC DNA, ligated into SuperCos 1 and packaged into phage λ heads. This was termed the packaged DNA.

3.3.3.5 Titering the cosmid library

A 1:10 and 1:50 dilution of the packaged DNA was made in SM buffer. 25µl of each dilution was mixed with 25µl of the OD_{600} 0.5 LE392 host cells in an eppendorf microcentrifuge tube and let sit at room temperature for 30 minutes. 200µl of LB media was added to each sample and incubated for 1 hour at 37°C, shaking the tube gently every 15 minutes, to allow the expression of ampicillin resistance. The microcentrifuge tube was then spun for 1 minute in a microcentrifuge and the cell pellet resuspended in 50µl of fresh LB broth. The cell suspension was then spread on LB plates containing 50µg/ml of ampicillin, and incubated overnight. The optimum dilution of packaged DNA was then calculated from the resultant colony numbers and sufficient infections performed to result in a 10x coverage library (Chia 1982).

3.3.3.6. Screening of the cosmid library

Colony lifts of the cosmid library were performed using Hybond N+ circular nylon membranes (Grunstein 1975). Bacteria harbouring a recombinant vector were plated out as described. After growth overnight at 37°C a replica was made by gently laying a nylon membrane disc onto the plate surface and allowed to completely moisten on the plate. The membranes and plates were then marked using a sterile 21 gauge needle dipped in indian ink to enable the orientation of the membrane with respect to the plate to be subsequently determined. This process was repeated to enable duplicate filters to be made. The filter was then transferred to 0.5M NaCl/0.5M NaOH for 10 minutes to lyse host cells and denature DNA. After neutralisation for 10 minutes in 2M NaCl/0.5M Tris HCl pH8, the filter was rinsed in 2xSSC and baked in the microwave at high heat for 45 seconds. The membranes were then hybridised with labelled total human DNA and corresponding positive colonies were picked using sterile toothpicks and gridded onto LB-Amp plates and grown overnight at 37°C. Colony lifts were again made of these plates and were screened in turn, with labelled probes: LE12, ACH207 and the YAC ends: Sup4L and Sup4R. Corresponding positive cosmid colonies were then picked using a sterile wire loop and inoculated into 100mls of LB media containing 50μg/ml of ampicllin and incubated at 37°C overnight. Glycerol stocks were made of each of the cosmids selected in this manner and cosmid DNA preparations as per 2.3.1.1, were made of the remaining culture.

3.3.3.7 Construction of cosmid contigs

3.3.3.7.1 Fingerprinting of cosmids

Cosmid DNA was prepared following selection of the cosmid clones from the y26A8 cosmid library. DNA was digested with restriction endonucleases and the fragments were separated on 1% agarose gels and visualised with ethidium bromide. The restriction digested DNA was then transferred to a nylon membrane by Southern blotting. The restriction enzymes used were most often HindIII, EcoRI, BamHI and PstI. However others were selected according to the restriction map of y26A8. The restriction digested cosmid DNA could be visually compared from one cosmid to another, allowing cosmids with similar restriction fragment patterns to be grouped together.

3.3.3.7.2 Riboprobes for end-probes of cosmids

T3 and T7 ends of cosmids were isolated by probing restriction digested cosmid DNA, Southern transferred to nylon membranes, with end labelled T3 or T7 oligonucleotides (see 2.3.4.1). 500bp-3kb end fragments were selected from the restricted cosmid DNA and riboprobes were constructed using DNA-dependent RNA polymerase: T7 and T3 bacteriophage RNA polymerases. A 50µl reaction was used containing: 40mM Tris-Cl, pH7.5, 10mM $MgCl_2$, 5mM DTT, 2µg DNA template digested to completion with the appropriate restriction endonuclease (including the T3 or T7 phage promoter), 400µM dNTPs (dGTP, dCTP, dATP), 80µM dUTP, 50µCi[α-^{32}P] dUTP, 50µg/ml BSA and 10U RNA polymerase. This was incubated at 37°C for 30 minutes and the reaction was stopped by adding 2µl of 0.5M EDTA. The probe was then hybridised to (1)restriction digested cosmid DNA transferred to a nylon membrane and (2)colony lifts from the y26A8 cosmid library.

The cosmid contig order could be ascertained from the results of the riboprobe end probe and the fingerprint analysis of the restriction digested cosmids which allowed overlapping cosmids to be identified. New cosmid clones could be obtained from the cosmid library, using riboprobe end-probes of the most distal and proximal cosmids of the cosmid contigs. The riboprobes were also used to map the cosmids back to y26A8, to allow the orientation of the cosmid with respect to the chromosome 16 centromere.

3.3.3.7.3 Random primer labelling of end probes

Restriction fragments identified as end fragments of the cosmids from the y26A8 cosmid library were also labelled by random priming/primer extension (see 2.3.4.2). Cosmid DNA digested with a restriction endonuclease was separated on a 1% agarose gel and the appropriate fragment previously identified as an end fragment (3.3.3.7.2) and then labelled using the techniques described in 2.3.4.2. This labelled end fragment was used for the same purposes described in 3.3.3.7.2.

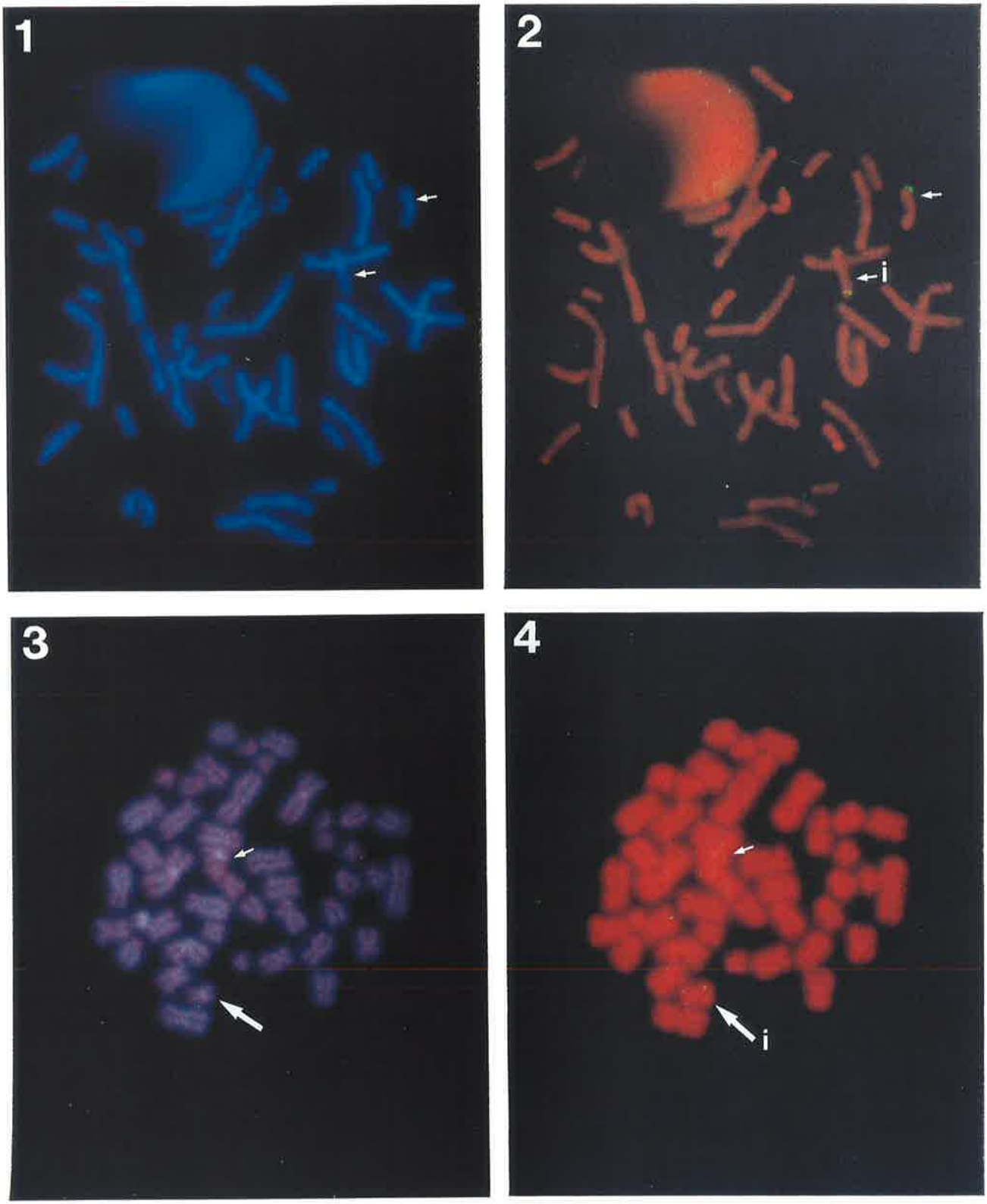
3.4 Results

3.4.1 Analysis of patient material by FISH using flanking markers

Metaphase chromosome spreads from 3 patients with inv(16) leukaemia were analysed by FISH using ACH207 and LE12 as proximal and distal probes (Smith 1992). The results of these experiments confirmed the positions of the two probes however in one patient, the fluorescent signal obtained from LE12 appeared split by the long arm breakpoint such that signal proximal and distal to the breakpoint was apparent with this probe (figure 3.2). This

Figure 3.2 Analysis of inv(16) leukaemia metaphase chromosomes by FISH

Metaphase spreads of leukaemic cells containing an inversion chromosome 16. A DAPI stain of each cell has been included to allow identification of the two chromosomes 16. The FITC labelled LE12 probe has been hybridised to the chromosomes using FISH techniques. In the upper photographs, a telomeric probe CRI-095 has been used to identify the portion of the long arm distal to the inversion breakpoint and in this case the two probes can be seen to remain on the distal portion of the long arm of the inversion chromosome 16 (arrowed and marked "i"). The normal chromosome 16 is on the periphery of the metaphase. In the lower photograph, no base probe has been used. The signal from LE12 can be clearly seen to be split on the inversion chromosome (large arrow).


1
2
i
3
4
i

result was interpreted to mean that either there existed a breakpoint cluster region which depending on the exact position of the breakpoint in each particular patient, could directly involve the LE12 probe or that there was cross hybridisation of LE12 with repeat elements contained within the DNA sequence of the long arm of chromosome 16. Southern analysis of HindIII, BamHI, EcoRI and PstI digested normal human and leukaemic DNA, probed with labelled LE12 probe, did not show any altered band sizes in the leukaemic patients when compared with normal controls. It was thus decided to proceed with positional cloning of the breakpoint on the premise that LE12 was positioned distally to the long arm breakpoint of the inv(16).

3.4.2 Sequencing of flanking genetic markers

Manual sequencing of the flanking DNA markers was undertaken as per the method outlined in 3.2.1. Satisfactory sequence was obtained initially from LE12 and ACH207. Three hundred base pairs were successfully sequenced and vector sequences excluded. From the human sequences obtained, oligonucleotides were synthesised for the purpose of PCR-based screening of the CEPH YAC libraries.

LE12:

Forward primer 5′ CGA GAT CCA ATC TGA GGC TTC CCT CTT T 3 ′

Reverse primer 5′ TAA GAT GCT TGT CAG AAT AGA GTC TGG A 3′

PCR product: 160bp

ACH207:

Forward primer 5' TCA TAT GTG GCA AGG ATT TGA GA 3'

Reverse primer 5' CAT CAC CAG GTC CTA ATG TTC TGG 3'

PCR product: 170bp

A further 900 base pairs of sequence were obtained for LE12 making a total of 1296bp and analysed for homology to known DNA sequences. Limited homology with TGFβ was found between nucleotides 730 and 780: 82.1% homology in 28 nucleotide overlap with human transforming growth factor β3 gene (figure 3.3). This was noted with interest, however no further homology was found in the extended sequence and any further sequencing was delayed until a YAC clone could be identified. Satisfactory sequence was then obtained for CRI-091 and CRI-02 and PCR primers were again designed.

CRI-091:

Forward primer 5′ TTA TGT GGC CCC TAA AAG CCA GGT ATT G 3′

Reverse primer 5′ AGC TTA CTT ATG CTT CGG CTC GTA TGT TGT GT 3′

PCR product: 200bp

CRI-02:

Forward primer 5′ TCC TCT AGA GTC GAC CTG CAG 3′

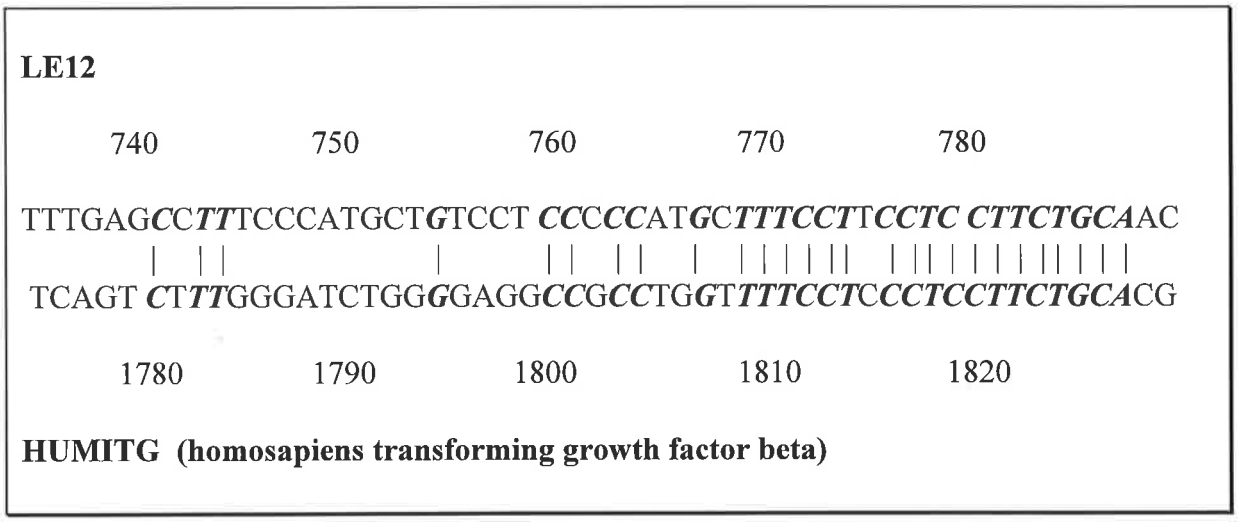
Reverse primer 5′ GGA TGT CCC GAG TTG GAA CAA 3′

PCR product: 220bp

3.4.3 Isolation of cloned YAC DNA positive for the flanking markers

Aliquots of the CEPH chromosome 16 YAC library were pooled for primary screening using the PCR primers described above. Positive pools were then secondarily screened for consistently positive PCR reactions (as per 3.3.2.1). These results were then forwarded to the CEPH laboratories along with primer aliquots and instructions for PCR conditions. Positive clones were then returned for quaternary checking following culture and DNA isolation. A

Figure 3.3 DNA sequence homology between LE12 and TGFβ



Sequence data for LE12 and homo sapiens transforming growth factor beta (HUMITGF) are shown. 82.1% identity in a 28 nucleotide overlap is demonstrated from nt. 760 to 788. Homologous nucleotides are in italics and bold faced type.

number of true positive YAC clones were received which were positive for the distal marker, LE12. The positive clones obtained (y50C2, y12F4 and y26A8) were then analysed by FISH for the correct positioning with respect to the long arm breakpoint and by Southern analysis for confirmation of the presence of the flanking marker LE12 and assessed for the presence of ACH207.

The approximate size of the human insert DNA of the YAC clones was assessed by pulsed field separation of the intact YAC DNA against suitable size markers as 200kb (y12F4), 385kb (y26A8) and 330kb (y50C2). YAC 12F4 was found also to be positive for ACH207, by Southern and by PCR. YAC 26A8 was not positive for ACH207 by PCR, nor on PFGE analysis of y26A8, however the results of some Southern analysis suggested an interstitial deletion involving at least part of ACH207 had occurred within this YAC. Results of the Southern transferred YAC DNA probed with LE12 and ACH207 are shown in figure 3.4. YAC 50C2 was positive for LE12 only, therefore extending the YAC contig distally. None of these YACs were positive for a 1kb unique fragment of the next distal probe, CRI-02. YAC clones positive for CRI-091 and CRI-02 were eventually received from CEPH 6 months after the receipt of the LE12 positive clones.

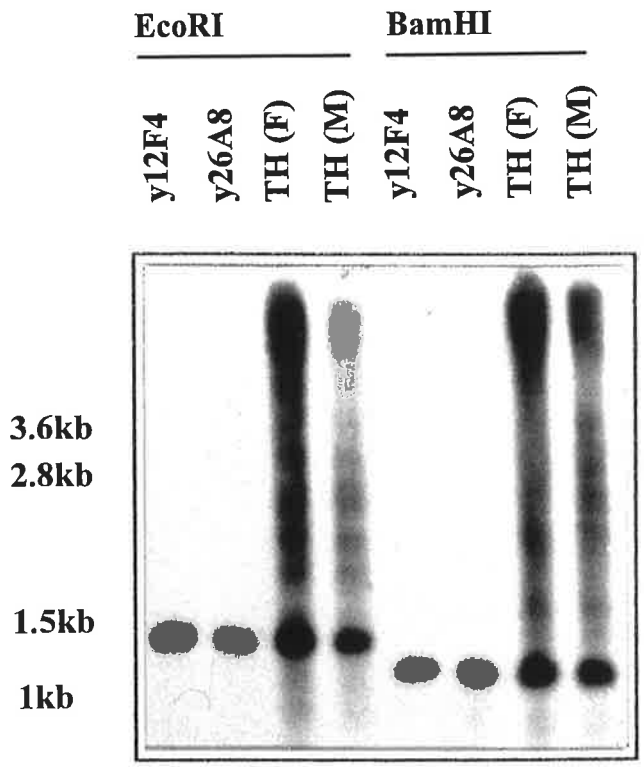
3.4.4 Restriction mapping of positive YACs

PFGE restriction mapping of 12F4 was commenced in the first instance. This YAC was initially chosen for analysis because it represented the smallest of the 3 YAC clones and was positive for the 2 flanking markers, LE12 and ACH207. However, with repeat culturing of the 12F4 clone, DNA extracted from the yeast colony failed to hybridise with LE12 and it became clear that a second YAC was contained within the same yeast colony and was being

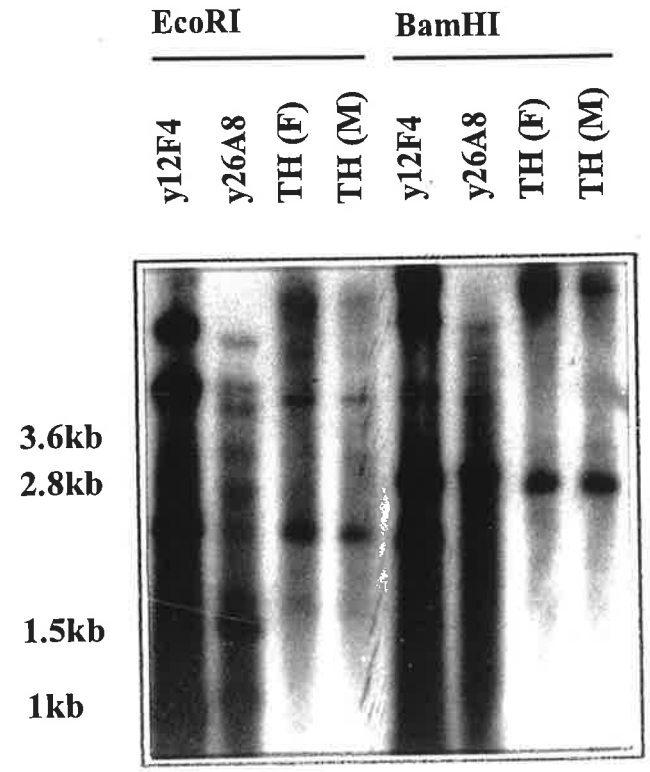
Figure 3.4 Southern analysis of YACs with probes LE12 and ACH207

Restricted YAC and genomic human DNA was hybridised to ^{32}P labelled probes, LE12 (A) and ACH207 (B). Autoradiographs of these experiments are shown. Corresponding 1.2 and 1.5kb bands are present in figure A, on the human and YAC DNA lanes for EcoRI and BamHI restriction endonucleases respectively. This is consistent with YAC 12F4 and 26A8 being positive for LE12. Figure B demonstrates corresponding bands for human DNA and YAC 12F4 restricted DNA when hybridised to labelled ACH207 probe. YAC 26A8 does not display the same hybridisation pattern, suggesting that a deletion may have occurred in the formation of that YAC, at the or near the position of ACH207.

A


EcoRI
BamHI
y12F4
y26A8
TH (F)
TH (M)
y12F4
y26A8
TH (F)
TH (M)
3.6kb
2.8kb
1.5kb
1kb

B


EcoRI
BamHI
y12F4
y26A8
TH (F)
TH (M)
y12F4
y26A8
TH (F)
TH (M)
3.6kb
2.8kb
1.5kb
1kb

preferentially selected. This second YAC was not positive for either of the two flanking markers but did contain human DNA as evidenced by hybridisation against total human DNA. On retrospective review of the pulsed field autoradiographs used for sizing the YACs, a faint signal using total human DNA as a probe, could be seen at 340kb, presumably representing the contaminating YAC. Yeast colonies of y12F4 containing only the YAC of interest could not be isolated from the contaminant, and eventually LE12 positive colonies could not be grown. This made y12F4 unsuitable for further analysis.

PFGE restriction maps were made of the remaining two YACs according to the methods outlined in 3.3.2.3, the results of which can be seen in figures 3.5, 3.6 and 3.7. These results confirmed the position of the probe LE12 on y26A8 and y50C2, localised to a 20kb NarI restriction fragment on y26A8 and a terminal-NarI restriction fragment approximately 20kb in length, on y50C2. The distance separating various restriction sites revealed a 22kb deletion had occurred in the formation of the y50C2 clone between the most distal SnaBI site on y26A8 and the next BSSHII site on the same YAC clone. This distance was estimated to be 44kb in y26A8 and 22kb in y50C2 (figures 3.6 and 3.7).

An interstitial deletion involving ACH207 had also occurred in y26A8 making it necessary to estimate the position of that probe from the minimal data achieved with y12F4. Considering that y12F4 was 200kb in length, the maximum distance separating the two markers was likely to be no greater than 200kb, if no interstitial deletions were present within y12F4. Both probes had already been linked on the same 460kb MluI PFGE fragment on digests of human DNA. A CpG island was found to be located toward the centromeric end of YAC 26A8

Figure 3.5 Pulsed field gel electrophoresis analysis of YAC DNA

DNA from y26A8 was separated on a PFGE Chef mapper. The autoradiographs of the resulting Southern transferred membrane can be seen in this figure. Figure A has been probed with labelled LE12 insert DNA and figure B has been probed with labelled pUC plasmid DNA. Unique bands are present in figure A which do not correspond to end fragment, vector positive bands. In particular a 20kb Nar I fragment is noted to be positive for LE12.

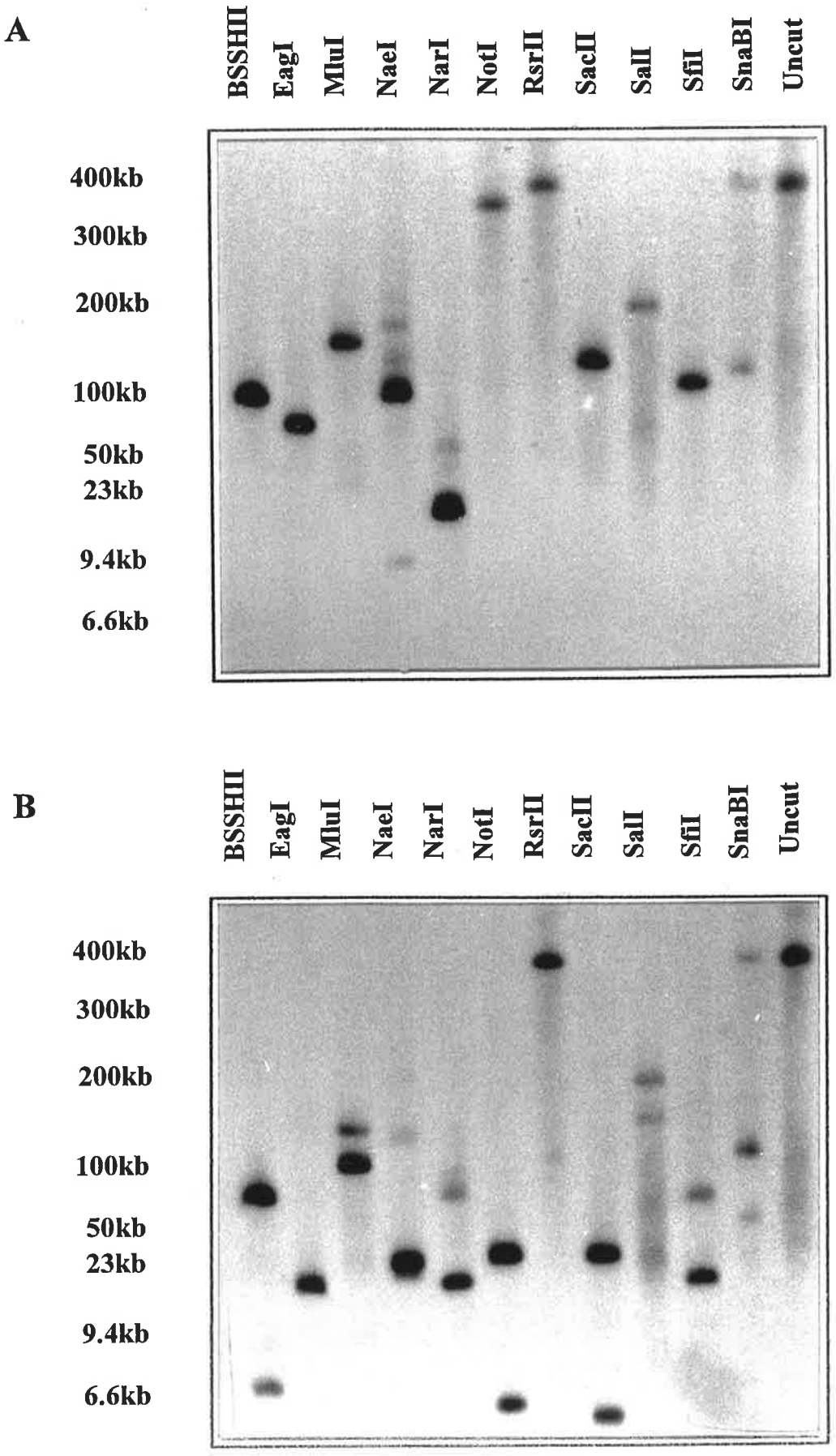

A
BSSHII
EagI
MluI
NaeI
NarI
NotI
RsrII
SacII
SalI
SfiI
SnaBI
Uncut
400kb
300kb
200kb
100kb
50kb
23kb
9.4kb
6.6kb
B
BSSHII
EagI
MluI
NaeI
NarI
NotI
RsrII
SacII
SalI
SfiI
SnaBI
Uncut
400kb
300kb
200kb
100kb
50kb
23kb
9.4kb
6.6kb

Figure 3.6 Pulsed field restriction map of y26A8

Restriction maps were made of y26A8 and y50C2 and these are shown in figures 3.6 and 3.7. These results confirmed the position of the probe LE12 on y26A8 and y50C2, localised to a 20kb NarI restriction fragment on y26A8 and a terminal-NarI restriction fragment approximately 20kb in length, on y50C2. The distance separating various restriction sites revealed a 22kb deletion had occurred in the formation of the y50C2 clone between the most distal SnaBI site on y26A8 and the next BSSHII site on the same YAC clone. This distance was estimated to be 44kb in y26A8 and 22kb in y50C2. An interstitial deletion involving ACH207 had also occurred in y26A8.

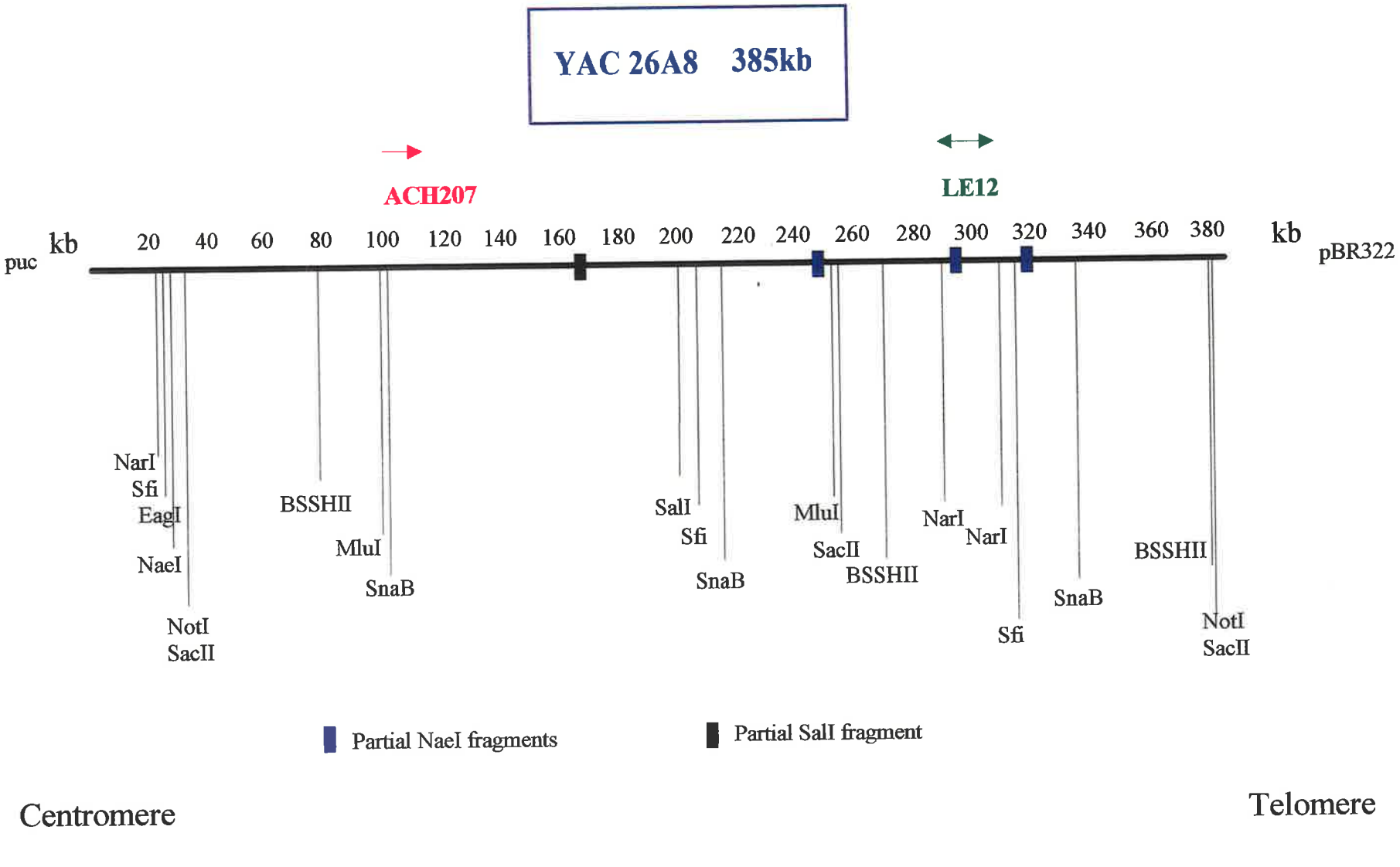
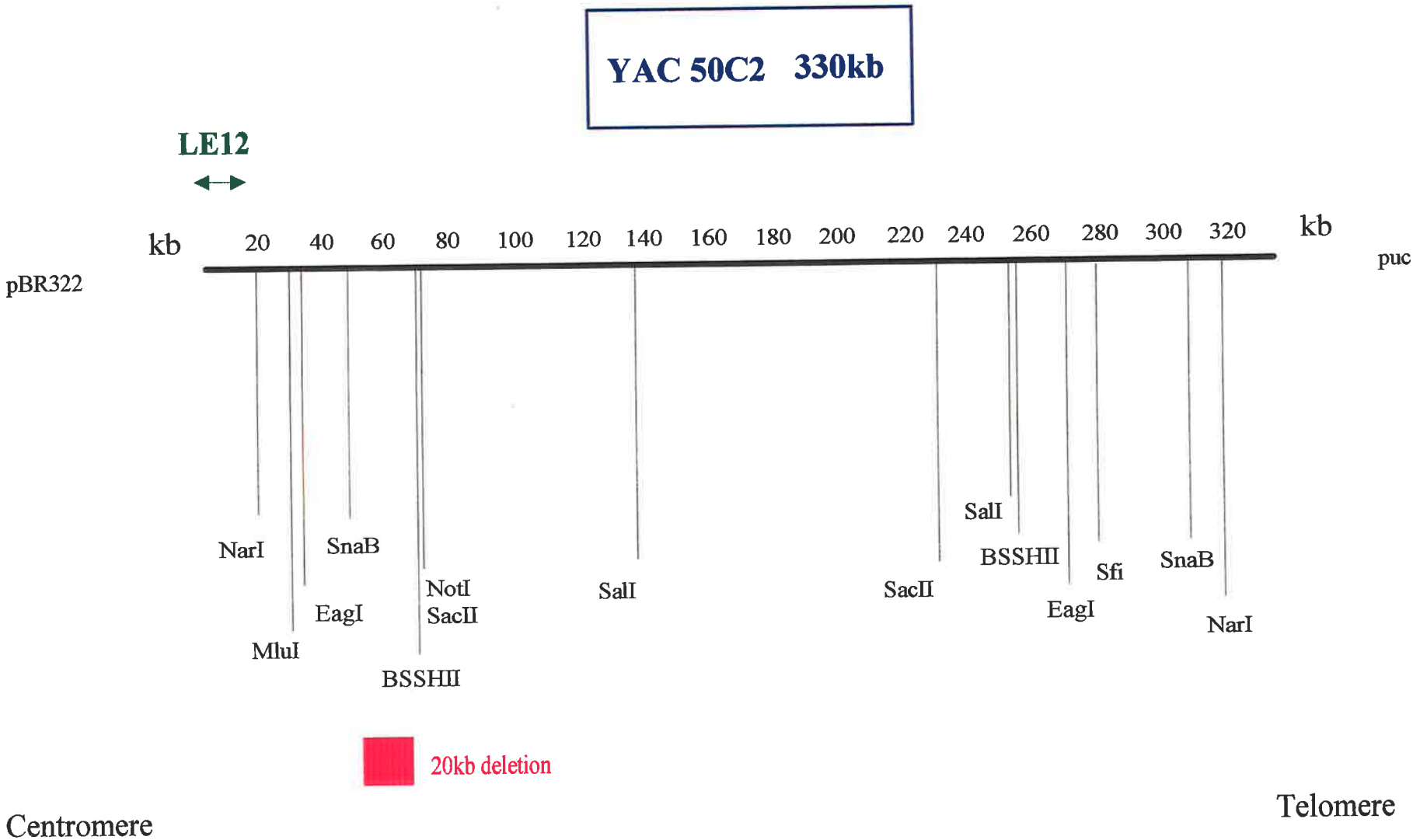

YAC 26A8 385kb
ACH207
LE12
puc
kb
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380
kb
pBR322
NarI
Sfi
EagI
NaeI
NotI
SacII
BSSHII
MluI
SnaB
SalI
Sfi
SnaB
MluI
SacII
BSSHII
NarI
NarI
Sfi
SnaB
BSSHII
NotI
SacII
Partial NaeI fragments
Partial SalI fragment
Centromere
Telomere

Figure 3.7 Pulsed field restriction map of y50C2

For legend see figure 3.6.


YAC 50C2 330kb
LE12
kb
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
kb
puc
pBR322
NarI
MluI
SnaB
EagI
NotI
SacII
BSSHII
SalI
SacII
SalI
BSSHII
EagI
Sfi
SnaB
NarI
20kb deletion
Centromere
Telomere

suggesting the presence of a possible house-keeping gene (Bird 1986), however no sequencing was performed at this time due to the fact that the centromeric end of y26A8 was likely to be at least 200-300 kb proximal to the long arm breakpoint of the inv(16).

3.4.5 *In situ* hybridisation of YACs to inv(16) chromosomes

In situ hybridisation against patient derived metaphase chromosome spreads containing the inv(16) chromosome was performed with y26A8 and y50C2 but not y12F4 in view of the contamination problems outlined above. Results of these hybridisations were disappointing in that in each case the YAC probes hybridised to satellite regions of D and G group chromosomes due to repeat sequences within the YAC and although the fluorescent signal appeared to be mostly proximal to the long arm breakpoint of the inv(16), fluorescent signal could be seen in a number of cells on both sides of the breakpoint. These findings made it difficult to be certain that the YAC clones crossed the long arm breakpoint of the inversion 16. Considering the Southern data confirming the presence of ACH207 and LE12 on y12F4 and the initial *in situ* results with the flanking markers, it was decided to continue analysis of the YAC clones, to subclone them and use the subclones to obtain more reliable FISH data.

3.4.6 Construction of cosmid contigs within the region of interest

A 10 times coverage cosmid library was constructed from y26A8 according to the methods outlined in 3.2.3. Colony lift membranes containing between 200 and 900 colonies each were hybridised against ^{32}P labelled total human DNA as an initial screening. Using LE12 as the initial internal probe, positive clones were then selected from the 10 times coverage library and were gridded onto new plates. Colony lift membranes from the gridded plates were then

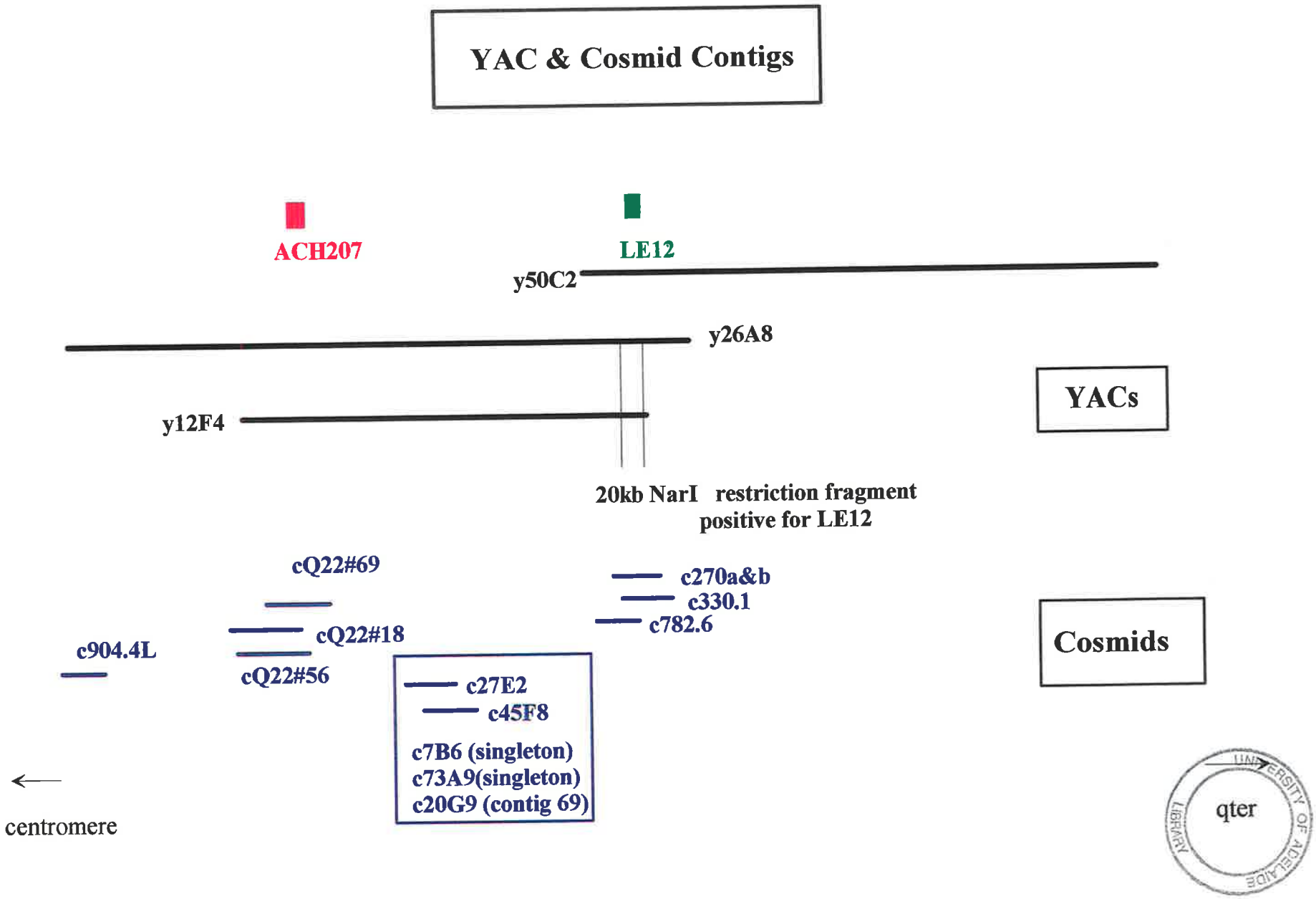
hybridised to ^{32}P labelled LE12 again and positive colonies were streaked out onto separate plates ready for DNA preparations to be made. LE12 was chosen as the initial internal probe due to the data achieved from the one inv(16) leukaemic patient (patient #2) where the fluorescent signal on FISH analysis of metaphase chromosomes had appeared split. This suggested that the breakpoint or breakpoint cluster may lie closer to the distal or telomeric end of the y26A8.

ACH207 and LE12 primers were also sent to Los Alamos for PCR-based screening of their cosmid library constructed from a flow sorted chromosome 16, derived from a mouse somatic cell hybrid containing human chromosome 16 as its only human chromosome. High density filters containing chromosome 16 derived cosmids from the Los Alamos library described above, were also screened using kinased oligonucleotides, comprised of the LE12 and ACH207 PCR primers. No LE12 positive cosmids were isolated however a cosmid contig (#432) positive for ACH207 was isolated and is shown in figure 3.8. Cosmid Q22#56 was labelled with ^{32}P and probed against membranes containing y12F4 and y26A8 and was found to map to the original y12F4 clone received from CEPH.

Cosmids mapped to the region of ACH207 by the Los Alamos laboratories, were obtained from the Los Alamos cosmid library and screened by Alu PCR for homology to the YACs. Five cosmids were identified: c27E2 and c45F8 forming a small cosmid contig (#173); cosmid c20G9, a member of contig #69; and two singletons: c7B6 and c73A9. These cosmids were obtained with the aim of bridging the deleted region of y26A8. DNA was

Figure 3.8 YAC and cosmid contigs positive for LE12 or ACH207

A diagram of the YAC and cosmid contigs positive for either LE12 or ACH207 is shown. This diagram is not drawn to scale. Cosmids beginning with cQ22 were positive for ACH207 and were obtained through the Los Alamos National Laboratories. Those cosmids within the insert (shown in figure) were mapped by Los Alamos National Laboratories to the region of ACH207 and were obtained to assist with contig construction. The LE12 positive cosmids and end cosmid c904.4L were derived from the y26A8 cosmid library.



extracted for the purpose of mapping by FISH and Southern however, results of FISH mapping with the most distal cosmids from the y26A8 was preferentially carried out.

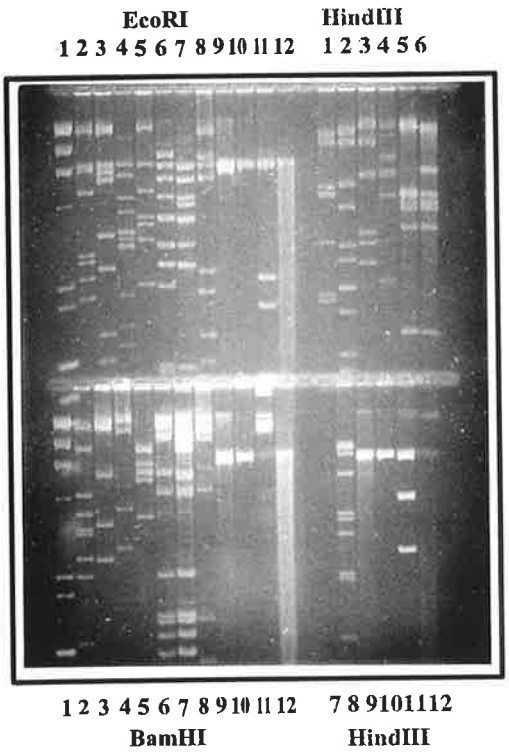
3.4.7 Restriction analysis of y26A8 cosmid library

Seven cosmid clones were selected by probing the colony lift membranes with ^{32}P labelled LE12: c330.1, c330.2, c564.1, c782.6, c904.2, c904.3 c904.4. These were purified and confirmed to be positive for LE12. Restriction digests and pulsed field separation of NarI, SfiI and SacII restricted cosmid DNA were performed to obtain fingerprints of the cosmids and assist formation of a cosmid contig. As previously stated, LE12 was found to be situated within a 20kb NarI fragment as shown in figure 3.6, and four of the seven cosmids selected contained this NarI fragment as analysed by pulsed field electrophoresis (c330.1, c564.1, c782.6, c904.4). The cosmids c782.6 and c330.1 also cut with SfiI, (SfiI was chosen due to the presence of an SfiI restriction site situated 5kb distal to the distal NarI site of y26A8, making these cosmids the most distally placed cosmids (figure 3.9).

Riboprobes of the T3 and T7 cosmid ends were made and hybridised to membranes containing restricted YAC DNA (refer 3.3.3.7.2). This allowed centromeric-telomeric orientation of the cosmids and construction of a cosmid contig from which cosmid clones could be selected for FISH analysis against inv(16) metaphase chromosomes. The most distally and proximally situated ends of the contig were used to select further cosmids from the library, for example the T7 end of 330.1 (the centromeric end) labelled by random priming and incorporation of ^{32}P, probed against the colony lift membranes of the y26A8 cosmid library, gave rise to c370a, c370b, c564a, c564b, c270a and c270b (Figure 3.9).

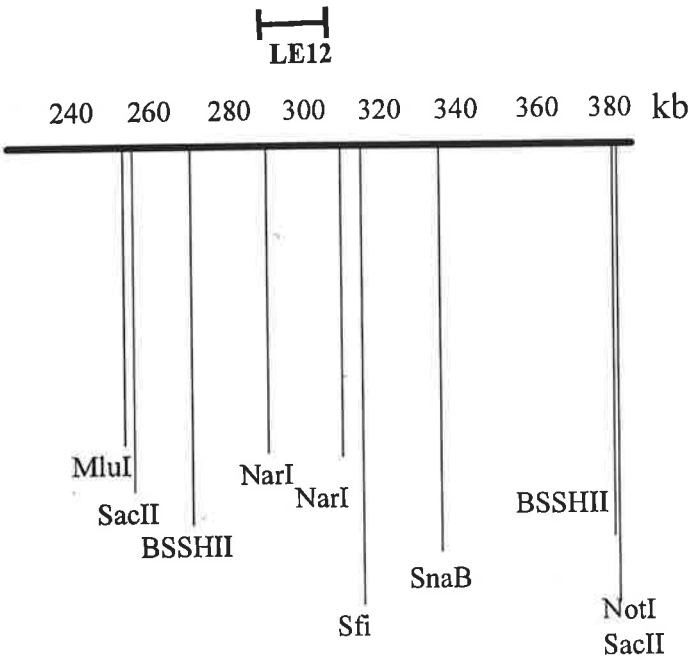
Figure 3.9 Fingerprinting of y26A8 cosmid library

DNA from cosmids derived from the y26A8 cosmid library were digested with the restriction endonucleases EcoRI, HindIII and BamHI. The agarose gel electrophoretic separation of restriction fragments is shown. Lanes 2-12 represent cosmids 270b, 370b, 564a, 564b, 782.6, 904.3, 330.1, 3T3, IIA1, IIA2 and 4T3 respectively. Lane 1 is the DNA marker, drigest. Several of the latter cosmids failed to cut with the restriction enzymes used (lanes 9,10 and 12). This occurred with all cosmids selected from the cosmid library using end-probe fragments of cosmids 330.1 and 782.6, to extend the cosmid contig distally.



Cosmid Digests

Cosmids



yac 26A8

Centromere

Telomere

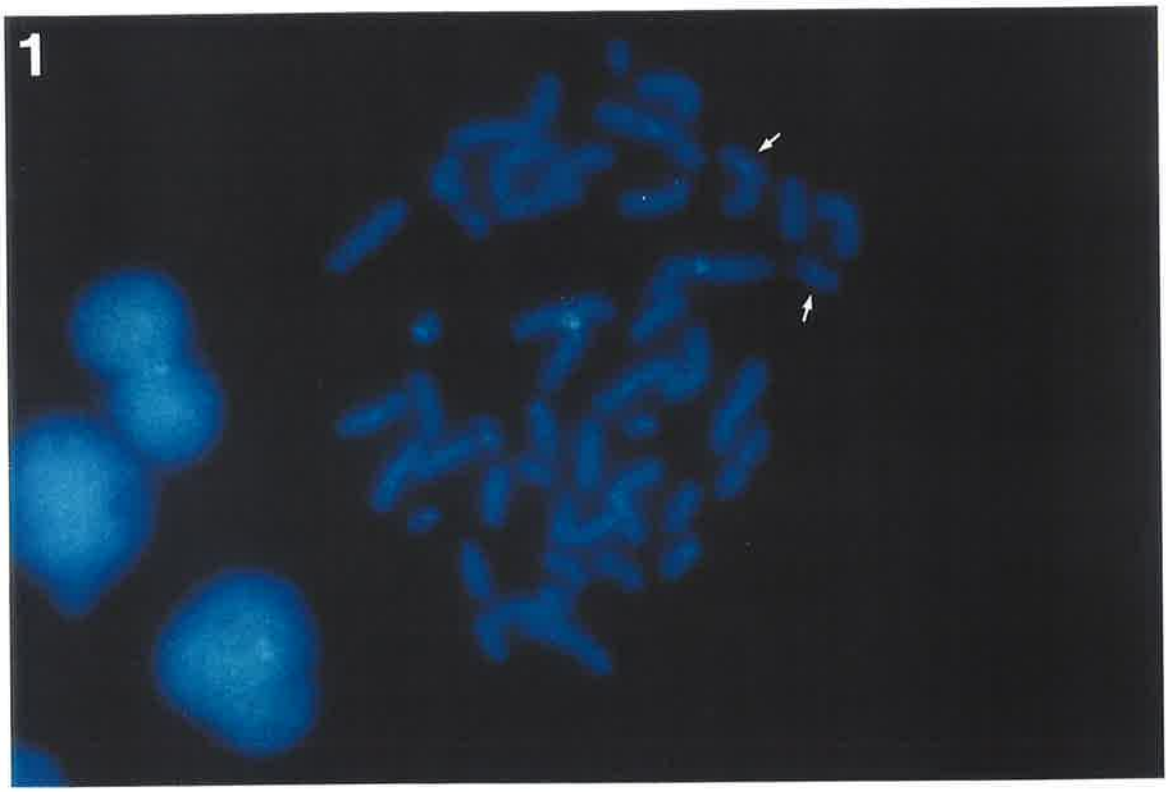
However, the T3 end or telomeric end of c330.1, using the same methods, failed to select colonies from the 10 times coverage y26A8 cosmid library, that contained human insert sequence. A cosmid contig spanning approximately 55kb was constructed and this covered the region of YAC 26A8, positive for LE12, however despite repeated hybridisations of the colony lift membranes, no colonies could be isolated to extend the contig beyond 10kb distal to the LE12 positive 20kb NarI fragment (figures 3.8 and 3.9). Almost all cosmid clones ended within 10kb of the distal NarI restriction site.

3.4.8 *In situ* hybridisation and Southern analysis of candidate cosmids

Cosmids 330.1 and 904.7 were selected for *in situ* hybridisation against inv(16) chromosomes as they represented the most distal and proximal cosmids respectively, of the y26A8 derived LE12 positive cosmid contig. Despite being positive for LE12, the marker previously found to be distal to the long arm breakpoint of inv(16), these two cosmids appeared to be proximal to the long arm breakpoint (figure 3.10). Southern analysis of EcoR1, BamHI and HindIII restricted leukaemic and normal human bone marrow DNA, probed with ^{32}P labelled pre-reassociated cosmid DNA (c330.1), failed to show any band shifts peculiar to the leukaemic samples (figure 3.11). These data suggested that the original in situ data concerning LE12 may have been incorrect. The LE12 FISH hybridisations were repeated under the same conditions previously used and against the same group of patients and were found to not to be reproducible, in that LE12 now clearly appeared to be a proximal probe.

Figure 3.10 Metaphase analysis of leukaemic cells with probe c330.1

c330.1 was hybridised to metaphase spreads derived from inv(16) leukaemic cells. A DAPI stain is included to identify the two chromosomes 16. The telomeric probe CRI095 was also used to mark the portion of the long arm of chromosome 16 distal to the inversion breakpoint. Fluorescent signal can be seen in both arms of the inversion chromosome 16 indicating that the two probes have been physically separated by the inversion event (two arrows and marked "i"). This placed c330.1 proximal to the long arm breakpoint. The normal chromosome 16 is the lower of the two arrowed chromosomes.


1

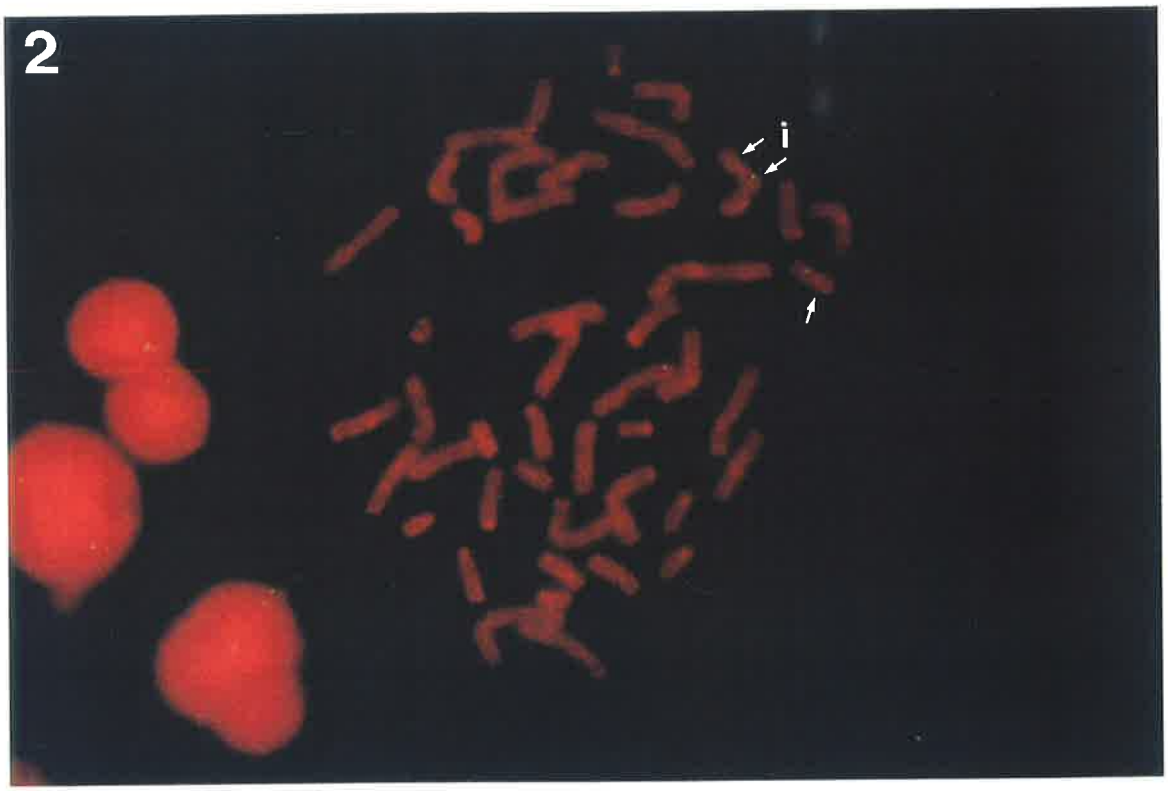
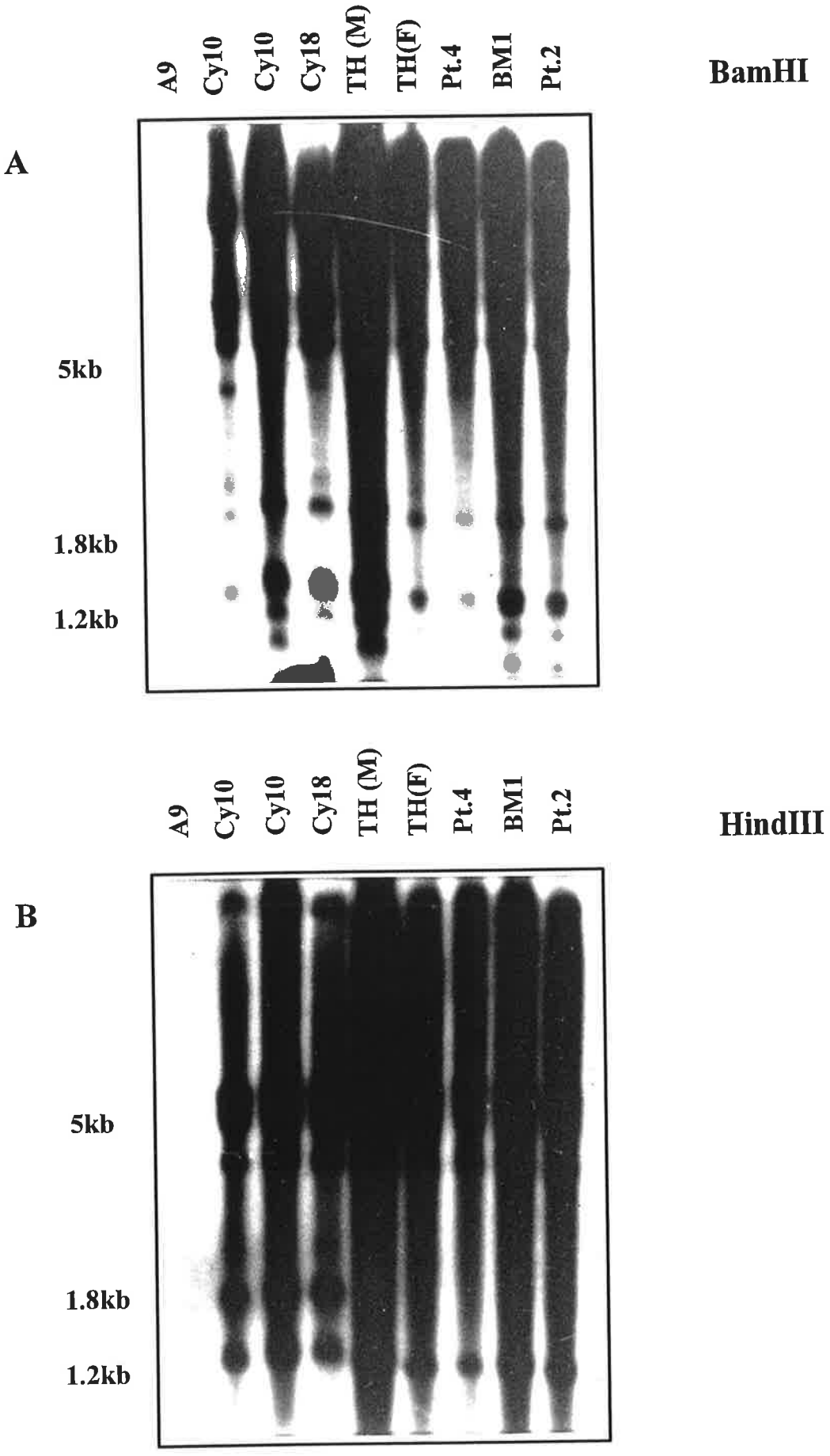

2
i

Figure 3.11 Southern analysis of leukaemic DNA probed with c330.1

Inv(16) leukaemic DNA was digested with the restriction endonucleases BamHI and HindIII. Autoradiographs of the membranes probed with c330.1 are shown. The somatic cell hybrid CY10 contains the inv(16) as its only human chromosome. DNA from CY18 and the parent mouse cell line, A9, are included along with normal total human genomic DNA for controls :TH(M) = total human DNA (male), TH(F) = total human DNA (female). Mouse-gene related bands are seen particularly in the HindIII digest above 1.8kb. No abnormally sized bands were seen in the leukaemic samples examined. This indicated that it was unlikely c330.1 crossed the long arm breakpoint of the inv(16).


A9
Cy10
Cy10
Cy18
TH (M)
TH(F)
Pt.4
BM1
Pt.2
BamHI
A
5kb
1.8kb
1.2kb
A9
Cy10
Cy10
Cy18
TH (M)
TH(F)
Pt.4
BM1
Pt.2
HindIII
B
5kb
1.8kb
1.2kb

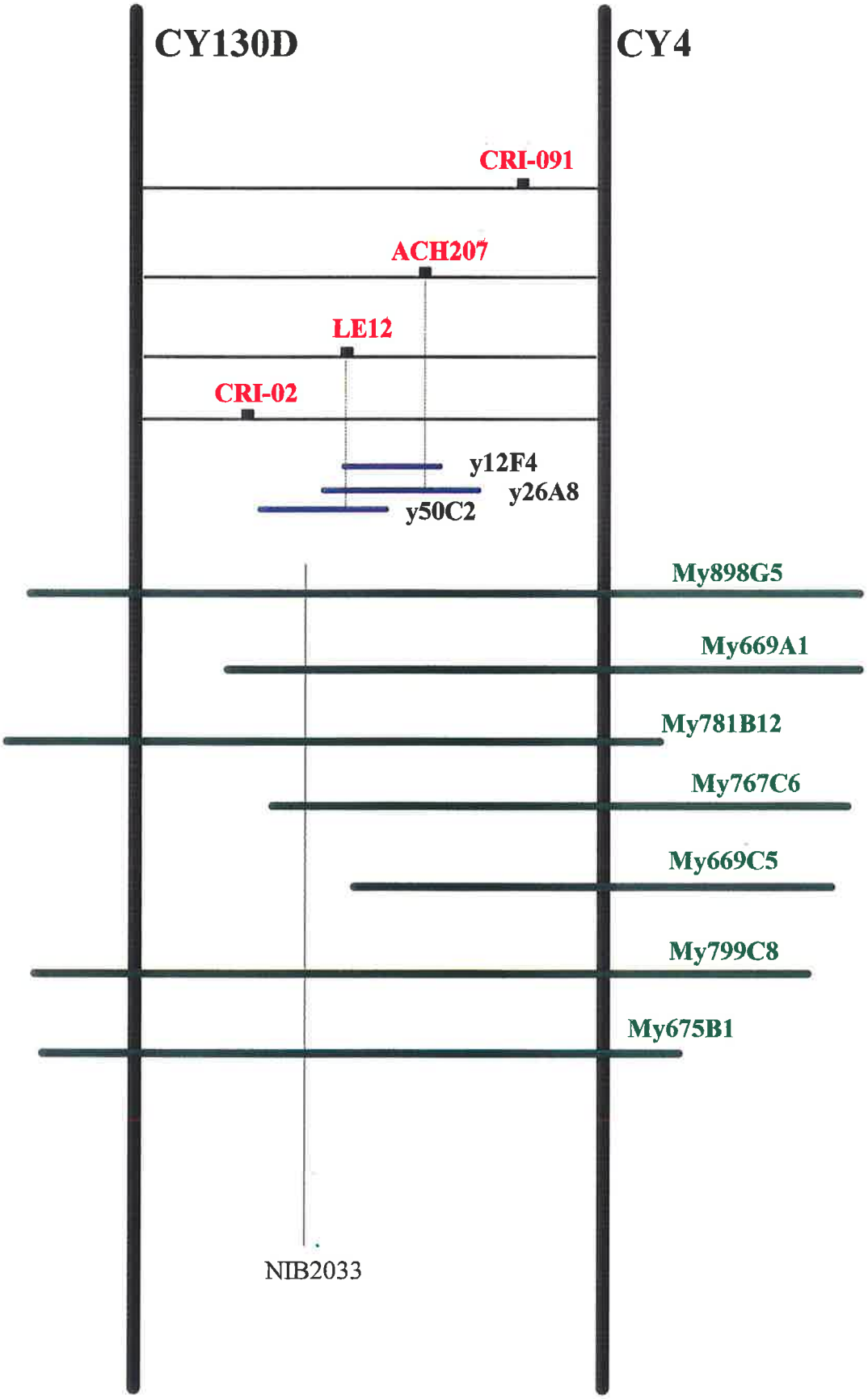
3.4.9 Core binding factor β (*CBFB*) cloned - comparison of cloned DNA

The breakpoint was cloned by Liu *et al* (1993), one month after establishing that the original FISH localisation of LE12 with respect to the long arm breakpoint of the inv(16) was incorrect. Probes which contained the long arm breakpoint of the inv(16) were obtained from Dr P.Liu which enabled checking of the YAC clones y26A8 and y50C2. Neither of the YAC clones proved positive for *CBFB* cosmids and neither of the *CBFB* cosmids (LA2.2 and LA4.1) were positive by PCR for the flanking markers. Human genomic DNA was restriction digested with rare cutting restriction enzymes and separated by pulsed field gel electrophoresis, transferred by Southern methodology and probed with a unique 0.7kb HindIII fragment from a *CBFB* cosmid (LA2.2) and a unique fragment from the LE12 containing cosmid 330.1. No linkage could be established by this method, between the two cosmid clones.

The estimated size of the CY130D/CY4 interval is approximately 2Mb. The distance separating the LE12 derived cosmid contig from the CBFB gene is likely to be of the order of 1Mb or less, however, an accurate assessment of this distance has not been possible. The two contigs can not be physically separated from each other using FISH techniques against metaphase chromosomes to estimate the distance separating the two cosmid contigs and PFGE proved unable to link the two cosmid contigs on a large restriction fragment. Considering the information available concerning the breakpoint genes, it was decided to terminate this phase of the project at this time rather than attempt to clone the intervening DNA between y50C2 and the genomic cosmids LA2.2 and LA4.1. Figure 3.12 demonstrates

Figure 3.12 Current map of CY130D to CY4

A current megaYAC map of the CY130D - CY4 interval is shown. This figure outlines the change in detail of the constructed chromosome 16 maps that have occurred over the last 2 years as a result of the human genome project. NIB2033 is a expressed sequence tagged site linking the megaYAC positions. The information in the top section of the diagram was established during the first results chapter of the thesis.


CY130D
CY4
CRI-091
ACH207
LE12
CRI-02
y12F4
y26A8
y50C2
My898G5
My669A1
My781B12
My767C6
My669C5
My799C8
My675B1
NIB2033

the change in information available in the interval CY130D - CY4 from then until the current day. Only megaYACs are shown.

3.5 Conclusion and discussion: Relevant literature review

3.5.1 Discussion of data presented

The data presented in this chapter represents a standard positional cloning strategy for genetic breakpoints. Similar strategies were employed by the two groups who were successful in cloning the breakpoints viz. Liu (1993) and Dauwerse (1993). They had both initiated their search with the short arm gene (*MYH11*) and in the case of Liu, were able to "jump" to the long arm gene (*CBFB*) using an expression library constructed from an inv(16) cell line ME-1 (Yanagisawa 1991). This cell line was unavailable to us.

The fundamental strategy employed for cloning of the long arm breakpoint was in itself logical and reasonable however the approach was dependent on a single FISH result which was subsequently found to be incorrect. The inclusion of more than one DNA marker distal to the long arm breakpoint would have been ideal. At that time, the only other probe localised to the CY4-CY130D interval by FISH, which produced fluorescent signal distal to the long arm breakpoint, also showed signal proximal to the long arm breakpoint, making its position with respect to the breakpoint unreliable. In retrospect, the inconclusive results with the FISH using y26A8 and y50C2 positive for the distal of the two flanking markers (LE12), could have been interpreted as evidence that these YACs did not cross the breakpoint. However, this was only able to be confirmed by subcloning the y26A8 and producing cosmids distal to the breakpoint. Other strategies for finding cosmids localised to the

the region were used simultaneous to the subcloning of y26A8, however all of the cosmids isolated, were positioned proximal to the LE12 positive y26A8 derived cosmid contig.

The most likely explanation for the incorrect positioning of the LE12 marker is the presence of specific low abundance repeat DNA sequences in the region of the long arm of chromosome 16. Chromosome 16 contains low abundance chromosome 16 specific repeats (LAR), some of which have been sequenced by Stallings (1992b) and localised to 16q22.1 and 16p13.13, the bands containing the inv(16) breakpoints. They describe the repetitive sequences as possessing similarity to the M13 minisatellite motif which appears on all human chromosomes. The LAR sequenced by Stallings contains a 40 base pair sequence repeated four times. These repeat sequences tend to cause problems with linear contig formation due to false positive overlaps in the assembly of cosmid contigs. Although these repeat sequences do not directly affect the long arm breakpoint of the inv(16), a paper by Dauwerse (1992), provides information concerning several blocks of chromosome 16 specific repeats and suggests that extensive cross homology between the long and short arm of the chromosome 16 facilitates the inversion and translocation events. This was subsequently found to be irrelevant to the DNA sequence directly involved with the inv(16) breakpoints. There are additional local repeat elements within chromosome 16 particularly within the region of Fra16B which is located in the same interval as the inv(16) long arm breakpoint which cause false interpretation of hybridisation around this site (E. Baker, personal communication). It is therefore highly likely that LE12 possesses repeat elements localised within the 16q22.1 region which caused the localisation of LE12 to be misinterpreted in the original FISH experiments. LE12 was hybridised against restricted human DNA which had been cut with a

number of restriction enzymes (EcoRI, HindIII, BamHI, PstI, NotI, MluI, SacI, SacII, NarI, SfiI, BSSHII and others), however no duplication or dual signal could be found using standard hybridisation conditions which could be consistent with the presence of a repeat containing element.

It became clear that the original localisation of LE12 distal to the breakpoint was incorrect, once cosmids positive for LE12 clearly mapped proximal to the breakpoint. The next distal marker CRI-02 lies an uncertain distance from LE12 and from the breakpoint. YACs positive for CRI-02 were eventually received following the screening of the CEPH YAC library, however it was decided not to further analyse these YACs as it would be a repetition of the work of Liu (1993).

A considerable chromosome "walk" may have been necessary to reach the inv(16) long arm breakpoint. Use of YAC clones more easily achieves a chromosome "walk" of one megabase however the high incidence of YACs which are chimeric (40% in megaYACs) (Doggett 1995) and which possess interstitial deletions creates gaps in the cloned DNA with respect to human genomic DNA (Green 1991). There were several deletions found in the YACs within this study as well as a yeast containing 2 unrelated YAC clones. It was assumed that the YAC present in these yeast clones was being preferentially transcribed/replicated at mitosis, resulting in eventual loss of the 12F4 YAC containing the human DNA of interest.

There are also inherent problems in multiple subcloning of DNA since those regions which are unstable in vectors, are then found to be under-represented in the final library (Doggett

1995). This may be in some situations vector specific, however these problems may be encountered in both phage and cosmid library construction as well as YAC and mega YAC construction. Similar problems were encountered in the making of the cosmid library from y26A8 with regions of the YAC DNAnot being represented in the 10x library. This may be due to inherently unstable pieces of DNA due to highly repetitive regions or regions prone to deletion due to the replicative machinery of the vector being unable to replicate the region effectively (Doggett 1995). Currently, BAC and PAC vectors are being used and these appear to have less problems with deleted regions of DNA and the development of chimeric human DNA inserts.

The interval 16q22.1 appears to be a gene rich area (Callen 1992) and it is possible that a housekeeping gene was cloned during this part of the project subsequent to the finding of a CpG island (Bird 1986) near ACH207 in the centromeric end of y26A8. This possibility was not further investigated at the time because it did not have immediate relevance to the original project. The cloned DNA isolated during this work was made available to another scientific group with an interest in the *CAVII* gene (carbonic anhydrase VII) located within the CY130D - CY4 region. The details of this work have been forwarded to the Los Alamos National Laboratories to assist with their collaborative work in cloning the human chromosome 16 and the construction of cosmid contigs and DNA maps within the region cloned.

3.5.2 *CBFB* and *MYH11*

3.5.2.1 Breakpoint and transcript characterisation

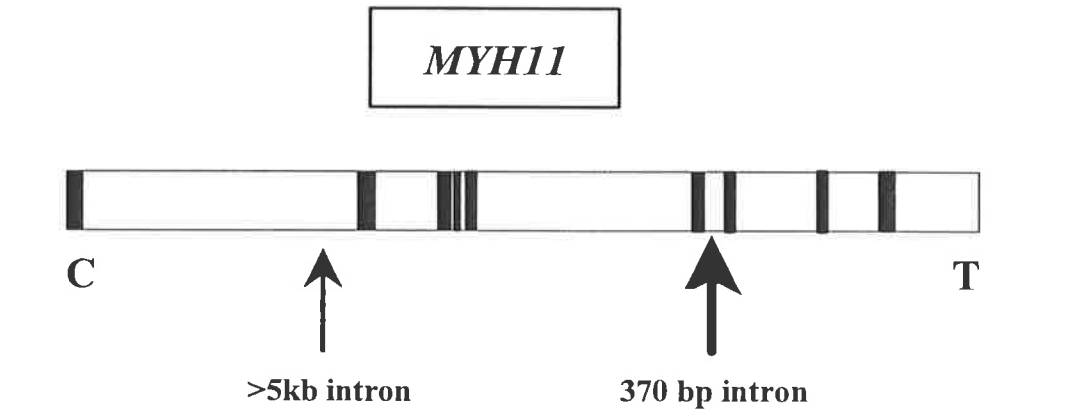
The breakpoints of the inversion chromosome 16 associated with M4Eo were cloned (Liu 1993, Dauwerse 1993) and shown to involve fusion between the gene encoding a unique transcription factor, *CBFB*, present on the long arm of chromosome 16, and the gene *MYH11* on the short arm. The genomic structure of the *CBFB* has been determined and found to be very similar to the murine homologue (Ogawa 1993a, Liu 1993, Bae 1994). The gene spans 50kb of genomic sequence and contains 6 exons. It is highly conserved in animal species as distant as *Drosophila melanogaster* (Ogawa 1993a) and the exon intron boundaries, with exons ranging in size from 78bp (exon 1) to approximately 2kb (exon 6) and introns ranging in size from 571bp (intron 2) to 16kb (intron 5, the intron in which most of the breakpoints occur) are identical to those in the murine homologue. The CBFB promoter region has typical features of a housekeeping gene, including high G+C content, high frequency of CpG dinucleotides and lack of canonical TATA and CCAAT boxes. It also has a highly polymorphic CCG repeat in the 5′ untranslated region which is not present in the murine gene (Hajra 1995a). There is evidence of conservation between the human and mouse genes, of certain transcriptional regulator binding motifs including binding sites for Ets family members, Sp1 and Myc (Hajra 1995a). The *CBFB* and the *MYH11* genes are both transcribed from centromere to telomere implying that the fusions are, 5'-*CBFB/MYH11-3′* and 5'-*MYH11/ CBFB-3′* (Liu 1993).

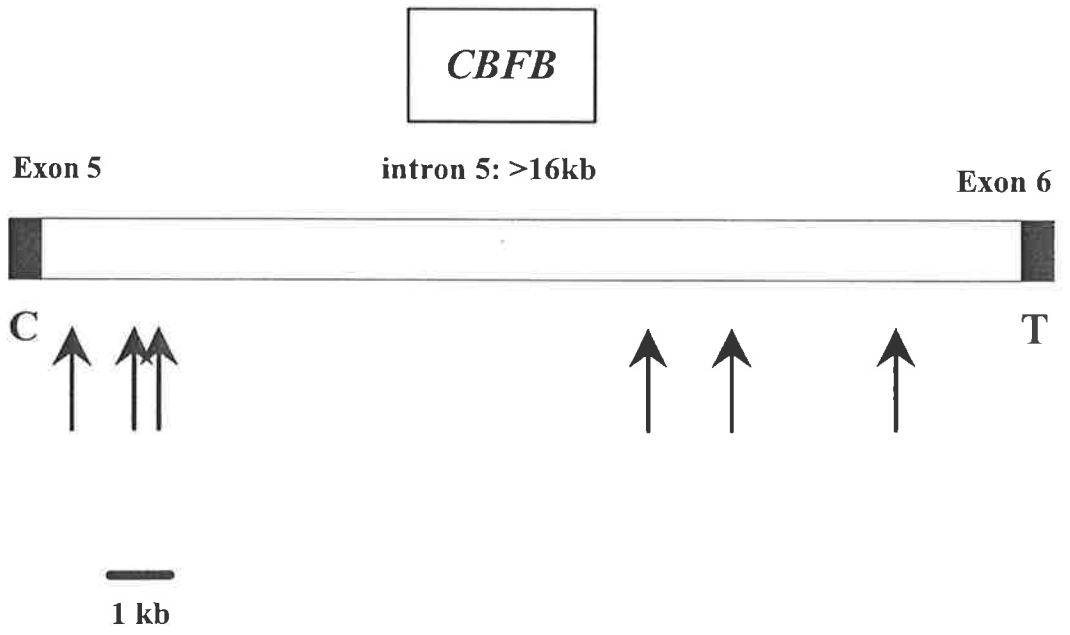
As stated, most long arm breakpoints seen in inv(16) leukaemias occur between exons 5 and 6 of the *CBFB* gene and Southern analysis of the long arm breakpoints indicates that these are

Figure 3.13 Breakpoints of the inv(16) chromosome

Diagrammatic illustration of inv(16) breakpoints at the genomic DNA level. Small arrows represent single breakpoints and the heavy arrow in the *MYH11* related diagram indicates the breakpoints in 5 patients. Filled boxes indicate putative exons for the *MYH11* and *CBFB* genes. (After *Liu, PP.* **Blood** 1995).

INVERSION 16 BREAKPOINTS:
position in 6 patients





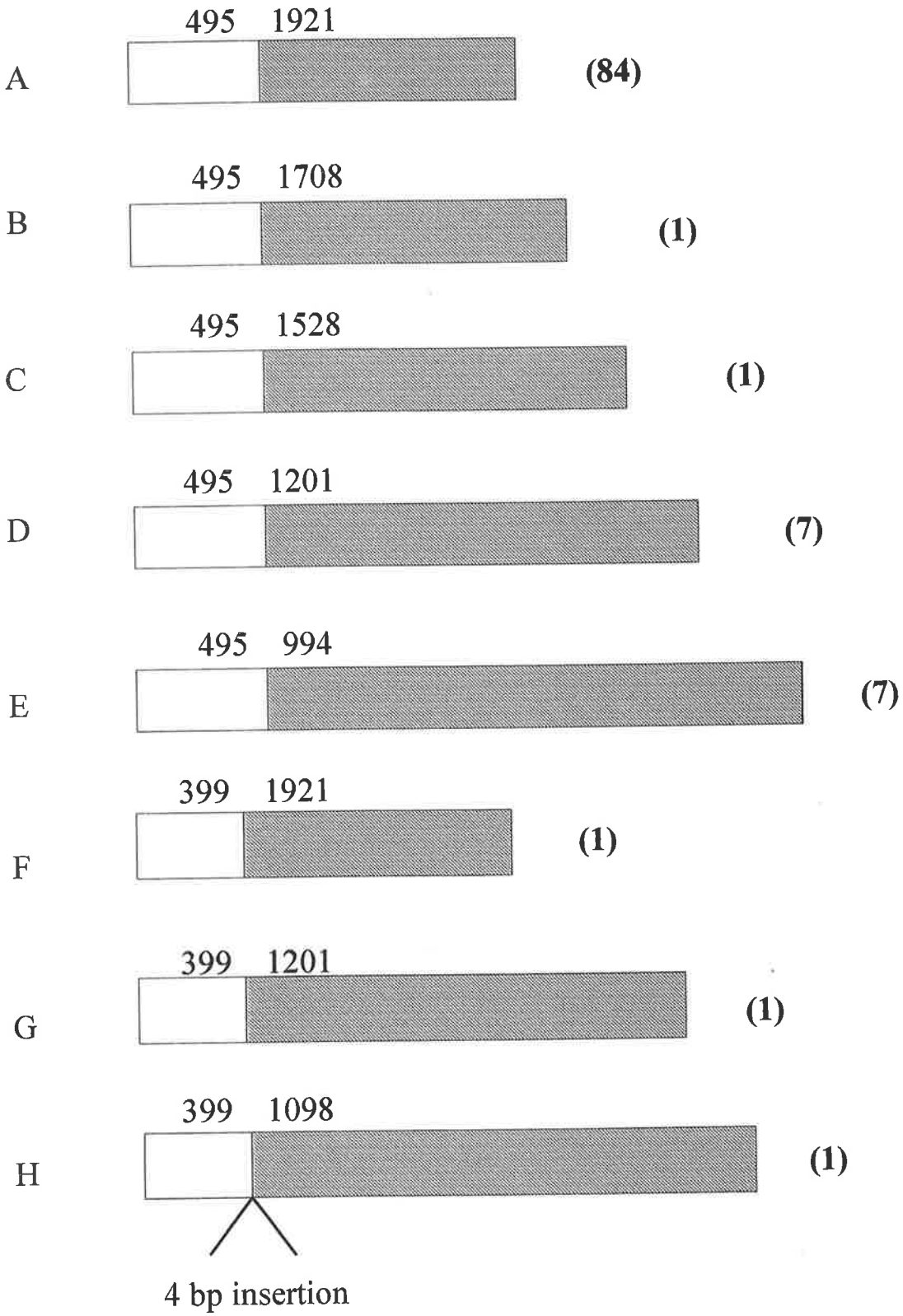
randomly distributed throughout intron 5 (Liu 1995). Figure 3.13 shows diagrammatic representation of the breakpoints sequenced to date. Intron 5 contains alternative splice donor sites 31 base pairs apart in the human *CBFB* and the murine *Cbfb* however the major PCR product detected in inv(16) contains only the shorter spliced form. The alternatively spliced transcript has been detected in a human striatum cDNA library and is 100% homologous to the transcript present in murine cDNA libraries (van der Reijden 1995). The alternative transcript results in a 187 aa protein compared to the 182 aa protein of the common splice form (Liu 1995) and alters the reading frame of exon 6 leading to different stop codons for the two transcripts. Multiple alternatively spliced transcripts have been observed for the murine gene and are expected for the human *CBFB* gene. The longer spliced form of *CBFB* would be out of frame for the *MYH11*gene reading frame if the common *MYH11* breakpoint had occurred. However, van der Reijden (1995), documented the alternative splice sites in exon 5 of the *CBFB* gene resulting in new fusion transcripts (types G and H figure 3.14) which fuse nucleotide 526 of the *CBFB* gene to nucleotides 1715 and 1921 of the *MYH11* gene respectively. The inclusion of an extra 206 nucleotides from the 5′ end of the *MYH11* gene in the type G transcript, restores the reading frame for the *MYH11* gene. The out of frame transcript seen in type H results in a stop codon located at the third codon after the *CBF/MYH11* junction resulting in a slightly truncated *CBFB* product.

In contrast to the *CBFB* breakpoints, the breakpoints in the *MYH11* gene on the short arm of chromosome 16, are found in a common breakpoint region which involves at least 3 different clusters of breakpoints within a 370bp intron as well as a number of other possible

Figure 3.14 *CBFB - MYH11* fusion transcripts in inv(16) leukaemias

Schematic representation of the 5′ CBFB-MYH11 3′ fusion transcripts resulting from the inv(16) leukaemic breakpoints. Primer pairs are designed around the breakpoint regions and used to confirm the presence of hybrid transcripts in leukaemic patients by RT-PCR. Numbers denote the nucleotides at the fusion breakpoints for the *CBFB* and the *MYH11* genes. The majority of patients are found to have transcript type A (84/103 patients), next common are types D and E. The transcript type has not been found to affect outcome in the patients studied. This data represents the compilation of data from Claxton (1994), Shurtleff (1995) and van der Reijden (1995).

CBFB/MYH11 Fusion Transcripts



breakpoints throughout the gene. Initially, 3 fusion points were detected for the *MYH11* gene: nucleotides 1921, 1201 and 994, all of which generated in-frame fusions enabling a fusion protein of 5′CBFB and 3′MYH11 to be generated. Subsequently, fusion breakpoints in the *MYH11* gene have been identified at nucleotides 1098, 1708 and 1715. However in the cases documented so far, 81.5% of the breakpoints are type A, that is occurring at nucleotide 495 in the *CBFB* gene and nucleotide 1921 in the *MYH11* gene. Diagrammatic representation of the fusion transcripts is shown in figure 3.14. The various sites of the *MYH11* breakpoints have no influence on the reading frame of the 5'-*CBFB/MYH11-3'* fusion mRNAs, of which ten different variants have so far been described (Liu 1995, van der Reijden 1995, Hébert 1995, Shurtleff 1995, Poirel 1995).

In most cases the fused 5′-*CBFB/MYH11-3′* sequences are precisely joined at exon intron boundaries, while in others, small insertions are apparent which still preserve the reading frame of the transcript (Shurtleff 1995). The majority of the *CBFB* gene is preserved in the fusion product losing only the last 17 or 22 amino acids from the C terminus of the coded protein, depending on alternative splicing of the *CBFB* gene. However, Shurtleff *et al* also reported the finding of three inv(16) patients in whom the *CBFB* breakpoint resulted in a fusion breakpoint at nucleotide 399 resulting in the retention of 133 N-terminal amino acids compared with 165 for the common fusion breakpoint. This may result in a different affinity for the α subunit of the CBF heterodimer, the functional significance of which is discussed in the next section. The C terminus of the fusion protein is provided by varying amounts of the carboxy terminal portion of the *MYH11* tail fused to the *CBFB*.

In both the original cloning papers, a subgroup of patients with an associated short arm deletion of *5'MYH11* sequences was described, although the profile of the transcripts as detected by RT-PCR (type A also being the most common transcript seen in this subgroup) was not altered (Liu 1993, Dauwerse 1993). A 5'-*MYH11/CBFB*-3' transcript has not been identified in either the deletion or no-deletion group suggesting that the generation of a fusion transcript at the short arm breakpoint is not critical in the development of the leukaemia, and that the 5'-*CBFB/MYH11*-3' fusion product is integral to inv(16) related leukaemogenesis (Liu 1993, Claxton 1994, Marlton 1995). The inv(16) and/or *CBF*β-*MYH 11* fusion transcripts have also been found in a number of other leukaemic subtypes including M1, M2, blastic transformation of CML and myelodysplastic syndromes implying that the leukaemogenic effects are not limited to *de novo* M4Eo (Claxton 1995, Shurtleff 1995). In several cases of t(16;16), a fusion transcript has also been documented suggesting that the molecular rearrangements of inv(16) and t(16;16) are equivalent (Claxton 1995). In two cases of cytogenetically diagnosed del(16)(q22) leukaemia examined (Liu 1995) one was found to contain the common type of *CBFB/MYH11* fusion mRNA. This may be due to misdiagnosis of a inv(16) cytogenetic abnormality for a del(16)(q22), due to the inherent difficulties in cytogenetic analysis encountered with chromosomes derived from leukaemic cells. The del(16)(q22) breakpoint has not been studied at the molecular level however it is presumed that in true del(16)(q22) cases, there is no involvement of the MYH11 gene therefore a different mechanism for leukaemogenesis would have to be contemplated.

3.5.2.2 Function and potential dysfunction of CBFB

From mouse studies, it is known that the core binding factor, CBF, also known as PEBP2 (polyoma virus enhancer binding protein), is a heterodimeric transcription factor comprised of α and β subunits (Bae 1993). The α subunit is encoded by a family of genes with homology to the *Drosophila runt* gene (Erickson 1992). *AML1* which is involved in the t(8;21) breakpoint seen in FAB M2 leukaemias (Miyoshi 1991, Nisson 1992, Erickson 1992, Nucifora 1994, Mitani 1994) encodes one of the *CBFα* genes (also known as *PEPB2αB*) (Bae 1993) and the β subunit is encoded by the *CBFB* gene, involved in the long arm breakpoint of the inv(16). The α subunit has trans-activational and DNA binding properties (Ogawa 1993b, Bae 1994) and binds to a core site in a motif present in the enhancers of many mammalian type C retroviruses. In particular the Moloney murine leukaemia virus (Mo-MLV): RACCRCA where R = purine (Speck 1987, Melnikova 1993) where it was critical for the T-cell specificity of the viral induced leukaemias (Speck 1990). This core binding motif is also present in cell surface proteins such as CD2, CD3ε, the enhancers of the T cell receptor genes (TCR), IL1R; cytokines such as IL3, IL5, GM-CSF, and G-CSF (Wang 1993). CBF has been shown to regulate the expression of T cell (Prosser 1992, Redondo 1992) and myeloid specific genes (Nuchprayoon 1994). *CBFB* contains no DNA binding motifs, and does not contain sequences homologous to any known protein (Wang 1992). Its function may be to stabilise the heterodimer complex formed with the α subunit (Ogawa 1993a, Wang 1993).

The role of this heterodimer in myeloid differentiation is not fully understood however certain interactions have recently been identified. It has been demonstrated that the

monocyte-specific expression of the receptor for the macrophage colony stimulating factor (CSF-1) is regulated at the transcriptional level by a monocyte specific complex (MonoB) which contains a member of the PEPB2/CBF family of transcription factors and is identical to or closely related to AML1 (Zhang 1994). Specific affinity of the monoB complex was demonstrated by competition assays including the consensus sequence for CBF binding (RACCRCA where R = purine) and the sequence for the Moloney murine leukaemia virus enhancer which both competed efficiently for the MonoB DNA binding complex. This nuclear factor/complex appears critical for the CSF-1 receptor promoter activity. CSF-1 (also known as M-CSF) stimulates the growth of a population of progenitor cells with a high predilection for macrophage maturation and induces progress of macrophage lines through cell cycle by modulating levels of specific cyclins (Sherr 1990, Stanley 1983). The CSF-1 receptor (the product of the *FMS* gene) is a tyrosine kinase which auto-phosphorylates (Sherr 1990). High levels of CSF-1 stimulate macrophage protein synthesis, cell division (Tushinski 1983 & 1985), and anti-tumour activity (Wing 1982) as well as other functions. It is conceivable that dysregulation of the gene would alter cell kinetics and proliferation while allowing some monocytic differentiation. In view of the association of the *AML1* gene with M2 leukaemias, and the *CBFB* gene with inv(16) and t(16;16) leukaemias, it is likely that the disruption of these genes are the leukaemogenic events in these leukaemias.

CBF is also involved in the regulation of murine myeloperoxidase and neutrophil elastase genes in immature myeloid cells (Nuchprayoon 1994). This study demonstrated that the CBF will bind and regulate the murine NE and MPO genes and that the murine NE enhancer contains additional functional elements, including potential binding sites for an Ets family

member and Myb. The Ets family member PU.1 has been shown to regulate the CD11b and macrophage colony stimulating factor receptor genes in monocytes (Pahl 1993, Zhang 1994). Two other studies have found binding sites for the Ets gene family and c-Myb in various parts of the T cell receptor enhancers in association with the CBF. Sun (1995) demonstrated that CBFα and three Ets proteins transactivate both the Mo-MLV and the mouse TCR β enhancer in transient expression assays and that transactivation by Cbfα requires both intact cbf and ets binding sites. Hernandez (1995) demonstrated functional synergy but independent binding of cMyb and CBF on adjacent sites in the T cell receptor delta enhancer. This may be of relevance to M4Eo/inv(16) leukaemias in that they have been associated with a specific immunophenotype with aberrant CD2 expression usually found on thymocytes, T cells and some NK cells (Adriaansen 1993, Paietta 1992). However, it is not known whether there are any other T cell specific features of the inv(16) leukaemias including evidence of T cell receptor rearrangement. The ligand for CD2, CD58, is also expressed on the leukaemic cells and the authors postulated that the interaction of these two molecules on cell-cell contact may have some positive effects on proliferation.

The role of the *MYH11* gene in leukaemogenesis is presently unknown. The oncogenic properties of the chimeric protein have been investigated using a 3T3 transformation assay (Hajra 1995b). NIH 3T3 cells expressing CBFβ-SMMHC chimeric protein acquired a transformed phenotype, as indicated by their ability to form foci, grow in soft agarose and form tumours in nude mice. No transformation occurred with normal CBFβ or the tail domain of the SMMHC suggesting that both elements of the chimeric protein are essential for transformation. Electrophoretic mobility assays showed that the chimeric CBFβ-SMMHC

protein formed a very large complex, most likely multimeric filaments mediated by the SMMHC carboxyl terminus. These complexes may interfere with the normal function of CBF in a dominant negative manner either by causing the CBFαβ complex to be mislocated in the cytoplasm of the cells or interfering sterically with adjacent binding of cooperating transcription factors (Lu 1995) such as cMyb (Hernandez 1995). Nuchprayoon postulated that the chimeric CBFB/MYH11 protein may bind with the α subunit through the truncated β subunit and the MYH11 component may allow the formation of an inactive tetramer. Lack of CBF function might prevent differentiation beyond the blast phase, similar to the PML-RARα oncogene in promyelocytic leukaemias.

It has been demonstrated that a mutant CBFβ containing only the N terminal 133 amino acids, equivalent to that present in the chimeric product involving the less common *CBFB* fusion breakpoint, has a reduced ability to interact with AML1 in an *in vitro* assay (Shurtleff 1995) when compared with a recombinant CBFβ containing the N-terminal 165 amino acids. This latter recombinant represents the number of *CBFB* amino acids coded for by the type A or common chimeric CBFB/MYH11 transcript. This suggests that different CBFB/MYH11 transcripts may have subtle differences in their biological activities. However, as yet no differences in the clinical outcomes of the patients with respect to their transcript type have been demonstrated (Liu 1995, Shurtleff 1995). This *in vitro* assay does not consider the effects of the SMMHC component on the protein-protein interaction and the effects of the multimers formed by chimeric CBF/MYH11 protein. Further studies are required to determine the exact role of *CBFB* in myeloid, particularly eosinophil and monocyte,

differentiation and the biological effects of the chimeric protein in the development of a leukaemic stem cell.

DELETION OF *MRP* IN INV(16) LEUKAEMIAS PROGNOSTIC IMPLICATIONS

Chapter 4

4.1 Introduction

As discussed in section 1.3, acute leukaemias associated with the inversion 16, are known to be part of a favourable prognostic group in AML (LeBeau 1983, Larson 1986). Inversion 16 leukaemias are in general chemosensitive, with a longer relapse free survival after induction chemotherapy, when compared with other acute myeloid leukaemias. The cloning of the two genes involved in the inversion breakpoints, *CBFB* and *MYH11*, and identification of the 5' *CBF/MYH11* 3' fusion transcript have not immediately explained the superior prognosis seen with the inv(16) leukaemias. However, little is currently known about the biological actions of the fusion transcript. The involvement of the *AML1* gene which represents the α subunit of the core binding factor, in the breakpoint of the t(8;21) as seen in M2 AML and the involvement of its functional partner, the *CBFB* gene or β subunit of the CBF, with the inv(16) leukaemias supports the hypothesis that the CBF transcription factor plays a role in early haematogenesis. It also suggests that an event which causes aberrant function of the CBF is leukaemogenic. It has been noted however that a group of morphologically identical leukaemias containing, as the sole chromosomal abnormality, a deletion restricted to the long arm of chromosome 16 [del(16q22)], are a separate prognostic group which respond less favourably to chemotherapy (Larson 1986). Taking into consideration that the molecular position of the del(16q) breakpoints may be variable, and may not directly involve *CBFB,* the difference in prognosis between these two cytogenetic subgroups raises questions concerning the specific roles of the genes involved in the short arm and long arm breakpoints of the inv(16) leukaemias with regard to other biological aspects of the leukaemia such as prognosis.

Both of the original reports of the cloning of the breakpoints in the inv(16) leukaemias noted that a subgroup of the leukaemic patients, included an interstitial deletion from the breakpoint at the 5′ end of *MYH11* (Liu 1993, Dauwerse 1993). A deletion was also noted in the preliminary pulsed field gel electrophoresis mapping of the short arm breakpoint of one leukaemic patient in this laboratory (Julie Nancarrow - personal communication). The deletion was approximately 300-400 kb in size. The finding of a large deletion associated with the inversion raised the possibility that other genes in addition to the CBFB and MYH11, may be disrupted by the inversion event and this may have implications for the biology of the disease.

The mRNA coding for a new multidrug resistance protein, MRP, was cloned from the human small cell lung cancer cell line, H69AR (Cole 1992) and the gene was mapped to chromosome 16 at band p13.1, the same region as that of the short arm breakpoint of inv(16) (Cole 1992, Slovak 1993). MRP, like P-glycoprotein, is a member of the ATP-binding cassette superfamily of transmembrane transporters. However, MRP is only distantly related to P-glycoprotein with 15% amino acid sequence homology. MRP has been shown in *in vitro* experiments, to confer resistance to the cell to a spectrum of structurally diverse, natural and synthetic products including drugs commonly used in the treatment of acute leukaemia, namely anthracyclines (Cole 1994). Overexpression of *MRP* mRNA and protein has been found in a number of multidrug resistant cell lines, including the human leukaemia cell lines, U937(drug resistant subline) (Slapak 1994) and HL60/ADR (Krishnamachary 1994). The *MRP* gene has not been shown to be amplified in leukaemias, although increased levels of *MRP* mRNA expression have been documented in *de novo* and relapsed leukaemia (Hart

1994, Schneider 1995) and chronic leukaemias (Burger 1994a). The mechanism for the increased expression is thought to be transcriptional activation or increased mRNA stability (Burger 1994b).

4.2 Aims of this Chapter

In this chapter it is hypothesised that the *MRP* gene may be deleted from the inversion chromosome 16 and that the presence or absence of the *MRP* gene on the inversion chromosome 16, and not the inv(16) alone, is an important prognostic indicator of the patient's response to chemotherapy. The aims of this chapter were to map the *MRP* gene more accurately using the high resolution chromosome 16 somatic cell hybrid panel and to map the gene in relation to the *MYH11* gene and the breakpoint cluster region of the short arm breakpoint of the inv(16). In addition, if *MRP* maps close to the MYH11 gene, to assess the effect of such a deletion on the response of the patient to chemotherapy and evaluate the overall effect this has on survival.

4.3 Materials and Methods (specific to this chapter)

4.3.1 Specimen collection and storage

A total of 22 patients with a histopathological diagnosis of AML and a bone marrow karyotype including the inv(16)(p13q22.1) were retrospectively studied. Cryopreserved bone marrow aspirates taken at diagnosis were morphologically classified confirming acute myeloid leukaemia according to FAB classification criteria and were karyotyped by routine procedures confirming the presence of the inversion chromosome 16. Table 4.1 provides clinical details, the karyotypes and white cell counts, at presentation where known. Patients

Table 4.1 Clinical details and karyotypes of inv(16) leukaemic patients

F = female. M = male. The age of the patient is given in years. FAB = French American British classification. The FAB subtype of AML refers to that outlined in figure 1.2. UM = unclassified myeloid leukaemia due to insufficient material. M2Eo refers to an M2 AML in which there is abnormal bone marrow eosinophilia. NA = sample not available for analysis. The summary of the MRP deletion status is included in this table for the entire 22 patients studied. Patients #1-4, #7,#10 and #12-22 were studied by FISH analysis of metaphase chromosomes. Where available, the survival data is included in table 4.4.

Patient (age,sex)	**Karyotype**	**FAB**	**WCC** ($x10^9$/L)	**MRP Status**
1 (16,M)	46,XY,inv(16)	M4Eo	104	deleted
2 (14,M)	46,XY,inv(16)	M4Eo	60	deleted
3 (25,F)	46,XX,inv(16)	M4Eo	25	deleted
4 (25,M)	46,XY/46,XY,inv(16)	M4Eo	60	not deleted
5 (58,F)	46,XX,inv(16)	M4	25	not deleted
6 (34,F)	46,XX/47,XX,+8,inv(16)	M1	2.7	not deleted
7 (48,M)	46,XY,inv(16)/ 47,XY,+22, inv (16)	M2Eo	267	not deleted
8 (51,M)	46,XY/46,inv(16)	M2	6.1	not deleted
9 (41,M)	47,XY,+8inv(16)/ 48,XY,+8,+22,inv(16)	UM	55	not deleted
10 (48,F)	46,XX/46,XX,inv(16)	M4Eo	36	mosaic
11 (18,F)	46,XX,inv(16)	UM	3.9	not deleted
12 (43,M)	46,XY,inv(16)	M4Eo	38	deleted
13 (35,M)	46,XY,inv(16)	M4Eo	116	deleted
14 (67,M)	46,XY/46,XY,inv(16)	M4Eo	NA	deleted
15 (41,F)	46,XX,inv(16)	M4Eo	NA	not deleted
16 (32,F)	47,XX,+8,inv(16)	M4Eo	NA	not deleted
17 (72,M)	46,XY,inv(16)	M4Eo	NA	not deleted
18 (32,F)	46,XX,inv(16)	M4Eo	NA	not deleted
19 (63M)	46,XY/46,XY,inv(16)	M4	NA	not deleted
20 (1,M)	46,XY,inv(16)	M4Eo	NA	deleted
21 (53,F)	47,XX,+8,inv(16)	M4Eo	NA	not deleted
22 (13,M)	46,XY,inv(16)	M4Eo	NA	not deleted

#5-9 and #11 were obtained from the SWOG Leukaemia Repository at the University of New Mexico. Patients #1-4, #10, #12 and #13 were collected from Australian sources whose bone marrow samples were cryopreserved at diagnosis and where possible at remission. All samples were stored in liquid nitrogen until required for analysis. Patients #14-22 were collected from Australian cytogenetic laboratories, and consequently no clinical data was available on these patients. Patients #1-13 were previously untreated except patient #8, whom was previously treated for a preleukaemic phase and patient #9 whom was in relapse following a complete remission obtained with Ara-C and Daunorubicin (regimen unknown). Ethics approval was obtained from the institutions involved in this study.

4.3.2 Somatic cell hybrids

A somatic cell hybrid (CY10) was constructed by Ms Sharon Lane in the Dept. of Cytogenetics at the Women's and Children's Hospital, Adelaide, by fusing the mouse cell line A9 with the bone marrow from patient 3 as per techniques described (Callen 1986). CY18 is a mouse/human somatic cell hybrid containing a normal chromosome 16 as the only human chromosome. The human chromosomes and cytogenetic breakpoints contained in CY19 and CY185 and other relevant hybrids are described in table 4.2. The *MRP* gene was mapped using a high resolution mouse/human somatic cell panel (Callen 1995).

4.3.3 DNA probes

4.3.3.1 Plasmid clones

MRP probes were obtained from S.P.C Cole and R.G. Deeley, Cancer Research Laboratories, Queen's University, Kingston , Ontario, Canada. *MRP*#14 is a 3.9 kb cDNA clone that spans

Table 4.2 Somatic cell hybrids used in the mapping of *MRP* to chromosome 16

Hybrid Line	Mouse Parent	Human Parent Rearrangement	Portion 16 Present	Other Human Present
Cy10	A9	inv(16)(p13.13q22.1)	inv(16)	few
Cy11	A9	t(11;16)(q21;p12.3)	p12.3→qter	absent
Cy18	A9	nil	complete 16	absent
Cy19	A9	t(13;16)(q12;p13.13)	p13.13→qter	yes
Cy158	A9	t(5;16)(q21;p11.2)	p11.2→qter	many, normal human 16
Cy185	A9	t(6;16)(p21.3;p13.12)	p13.12→qter	yes
Cy198	A9	t(1;16)(?q23;p13.2)	p13.3→qter	many

bases 110-4080 of *MRP* mRNA. *MRP*#7 is a 1.6kb cDNA clone that spans bases 1-1622 of MRP mRNA. These probes were isolated from a lambda gt11 H69AR cDNA library and subcloned into pGEM-3Zf(+) (Promega). (See figure 4.1). A 218bp unique probe derived from the 5′ portion of MRP#7 was identified by Ms. Ebba Kurz from Prof. Deeley's laboratory. LE12 (*D16S91*) is an anonymous 1.2kb EcoRI fragment, DNA clone which maps to 16q22.1, proximal to the inversion 16 long arm breakpoint.

4.3.3.2 YAC and cosmid clones

Dauwerse (1993), have constructed a yeast artificial chromosome (YAC) contig spanning the region of the short arm breakpoint cluster region of the inversion chromosome 16 and from one of these YACs (Y55ϕA) a cosmid library was generated. The vectors used in the subcloning were the pCpG cosmid vector (Dauwerse 1989) and the sCos1 vector (Evans 1989). Members of the cosmid contig derived from this cosmid library (figure 4.2) in particular zit79, zit14, zit132, and zit133, and a 2kb EcoRI MYH11 cDNA clone (LISP2) cloned into Bluescript were supplied by Dr. Martijn Breuning and Dr. Bert van der Reijden from the Department of Human Genetics, Leiden University, Sylvius Laboratories, Leiden, The Netherlands.

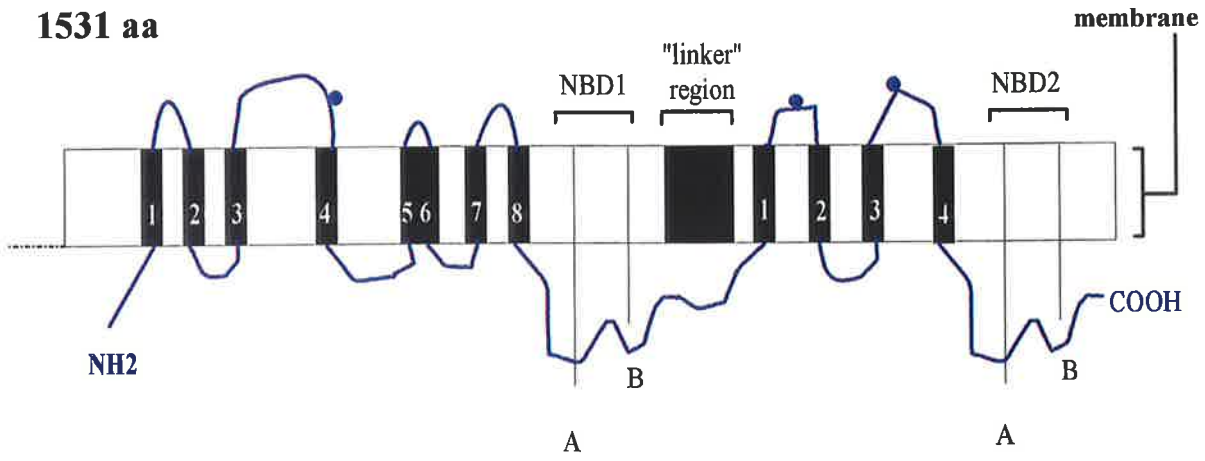
4.3.4 CEPH kindreds

The Centre d'Etude du Polymorphism Humaine (CEPH) families were used to study the inheritance of an allelic polymorphism in the 5′ untranslated region of the *MRP* gene. The CEPH reference family panel consists of families with large sibships, living parents and

Figure 4.1 Topological structure of MRP and positions of cDNA clones

A schematic diagram of the topological structure of MRP is shown. cDNA clones MRP#7 and MRP#14 are shown in relation to the coding sequence of *MRP*. MRP#7 includes the first 110 nucleotides (nt) of the 5′ untranslated region of the *MRP* gene extending to nt 1622. MRP#14 includes nt 110 to 4080. The full length mRNA for *MRP* is estimated at 6.6kb (Cole 1994).

Multidrug Resistance Protein



Coding Sequence

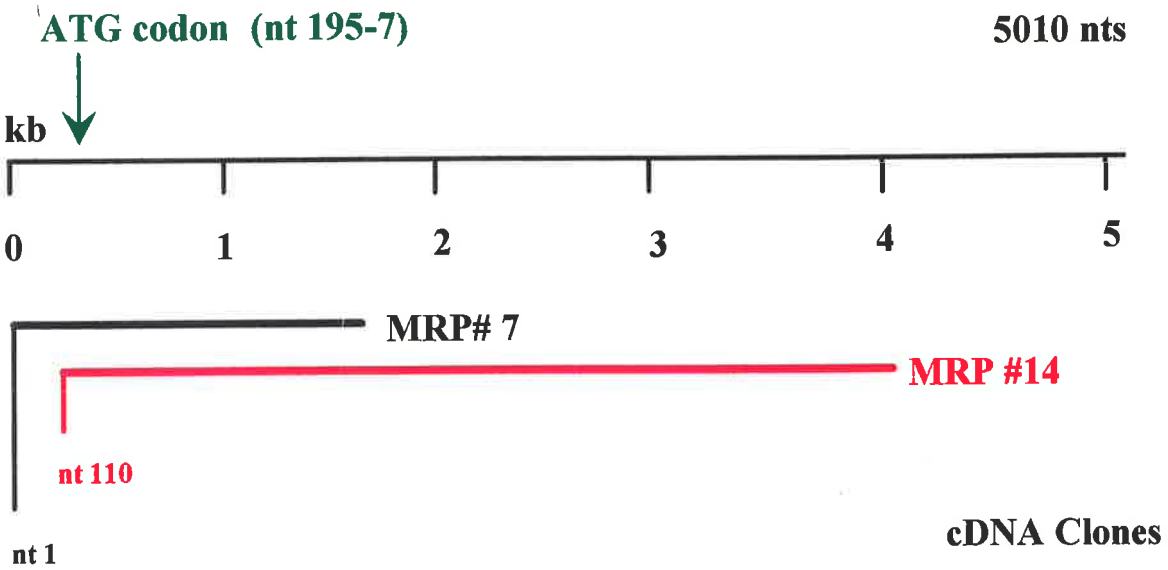
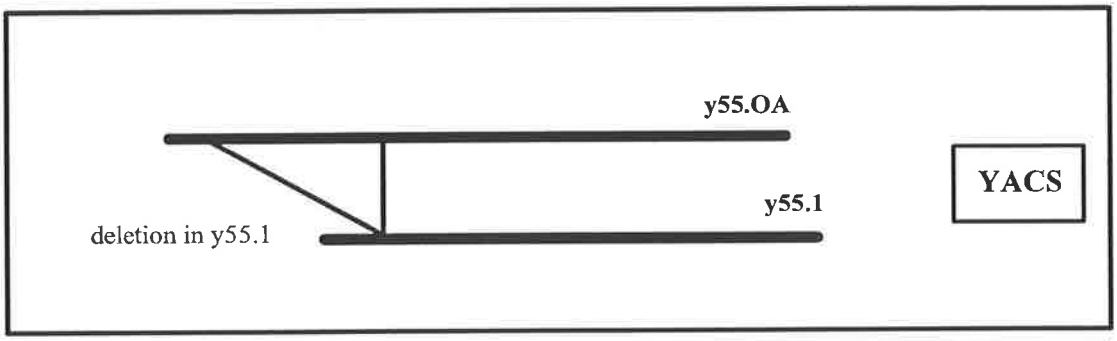
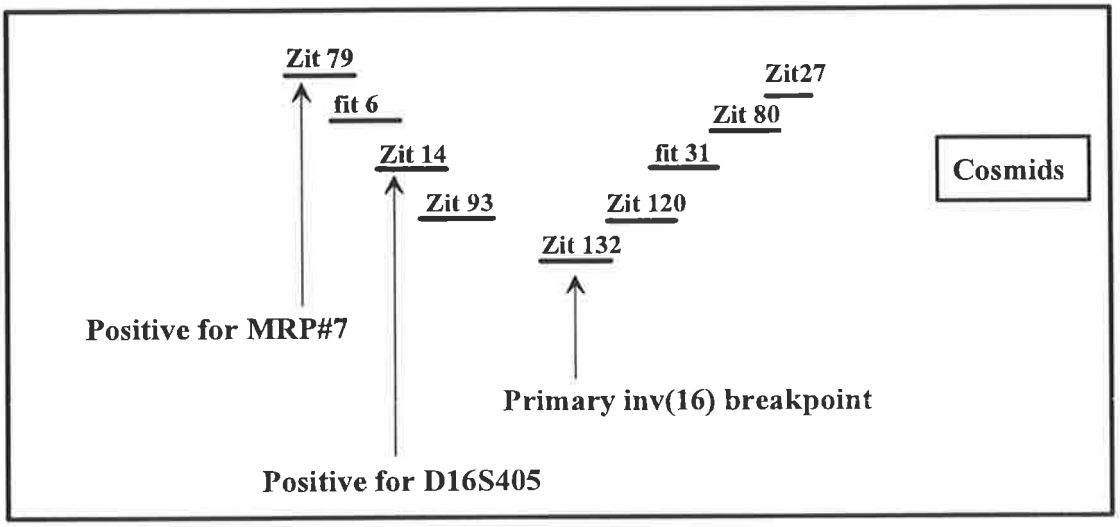
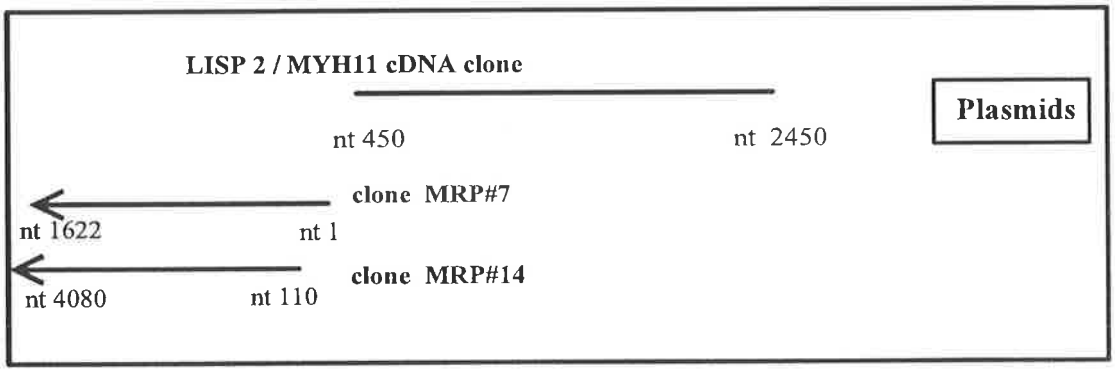


Figure 4.2 Genomic and cDNA clones of *MYH11* and *MRP*

Schematic diagram showing the relationships of the YAC and cosmid contigs containing the *MYH11* gene and the short arm inv(16) breakpoint region. Approximate distance separating cosmids zit132 and zit79: 150kb, represents the distance from the primary breakpoint region of *MYH11* to the 5' end of the *MRP*. zit79 is the most centromeric cosmid. cDNA clones for *MYH11* (LISP2) and *MRP* (MRP#7 and MRP#14) are shown. zit 79 is positive for a unique 218 bp fragment of MRP#7. zit 14 is positive for the AC polymorphic repeat, *D16S405*.


y55.OA
y55.1
deletion in y55.1
YACS


Zit 79
fit 6
Zit 14
Zit 93
Zit 132
Zit 120
fit 31
Zit 80
Zit27
Cosmids
Positive for MRP#7
Primary inv(16) breakpoint
Positive for D16S405


LISP 2 / MYH11 cDNA clone
nt 450
nt 2450
Plasmids
clone MRP#7
nt 1622
nt 1
clone MRP#14
nt 4080
nt 110

grandparents and is made available for the determination of genotypes for various DNA polymorphisms (Dausset 1990).

4.3.5 PCR amplification of the CCG repeat within the 5′ untranslated region

PCR primers were designed from the 5′-untranslated region of the published cDNA sequence of *MRP* (GenBank accession number L05628) (Cole 1992). These were used to physically map the *MRP* gene using the somatic cell hybrid panel. Primer sequences were:

MRP1F 5'TTGCGGCCCCGGCCCCGGCTCCCT 3'

MRP1R 5'AGCCATCGGCGCTGCAGAAGCCCCGGAG 3'.

Reaction conditions using 7-deaza-dGTP in place of dGTP and 2μCi α^{32}P(dCTP) (2.3.8 and 2.2.4), were as follows: *94°C 1min., 60°C 1.5 min., 72°C 1.5 min. - 10 cycles; 94°C 1min., 55°C 1.5 min., 72°C 1.5 min. - 25 cycles; 72°C extension cycle for 10 min.* The products were then heated to 95°C to denature the DNA and separated on a 5% acrylamide gel (refer 2.3.3.3.2). After drying the gel in a gel dryer (Bio Rad), the radioactive gel was then exposed to radiographic film for 12 -24 hours at room temperature, prior to developing.

4.3.6 Fluorescence *In Situ* Hybridisation

The *MRP*#14 probe was nick translated with biotin-14-dATP and hybridised *in situ* at a final concentration of 20ng/μl to metaphases from 8 patients with the chromosome 16 inversion. Metaphases were prepared from unstimulated short term bone marrow cultures by standard procedures (Smith 1992). The fluorescence *in situ* hybridisation (FISH) method was modified from that previously described (Callen 1990) in that chromosomes were stained before analysis with both propidium iodide (as counterstain) and DAPI (for chromosome

identification). Images of metaphase preparations were captured by a CCD camera (Olympus) and computer enhanced.

4.3.7 Microsatellite analysis by PCR amplification

Highly polymorphic (AC)n microsatellite markers mapping to the same interval as the MRP gene, as defined by the somatic cell hybrids CY19 and CY185, were used to demonstrate the deletion of a segment of DNA in that region. Primer pairs for *D16S49*(16ACCRI-0114), *D16S292*(16AC2.3), *D16S405*(AFM070ya1) and *D16S454* (16AC45G5) were used (table 4.3). All were highly informative polymorphisms with heterozygosities of 70-75%. Primer pairs designed to amplify these microsatellites resulted in products between 110 and 140 base pairs in length. Reaction conditions were used as per Shen (1991) and for each primer pair the thermocycler conditions were: *94°C 1min., 60°C 1.5 min., 72°C 1.5 min. - 10 cycles; 94°C 1min., 55°C 1.5 min., 72°C 1.5 min. - 25 cycles; 72°C extension cycle for 10 min.* Radiographic images were generated following drying procedures as in 2.3.3.3.2. These primers were used to generate PCR products from total human genomic DNA extracted from diagnostic bone marrow samples from patients with inversion 16 leukaemia and where possible from the remission peripheral blood and bone marrow samples of the same patients.

4.3.8. Assessment of gene dosage by quantitative analysis of DNA by Southern transfer and radioactive labelling of specific probes.

Genomic DNA (10μg) was extracted from the bone marrow cells of patients with inv(16) leukaemia at the time of diagnosis and where possible at remission. The DNA was digested to completion with BamHI restriction enzyme using a standard method (2.3.3.1), separated by

Table 4.3 Microsatellite primer sequences and repeat sequences amplified by PCR

Locus	Primer Sequence	Repeat Sequence
D16S49	(F) AATTCAAGGGAGGCTCATGTG (R) CACTCCTCCCTCTATGTTATG	$TGG(GT)_6$ $GGTGG(GT)_3$ $GG(GT)_{14}$ TCA
D16S454	(F) GCTGCCATCCTAAGCTTTGGTTAGG (R) AGGTCGACTCTAGAGGATCTTCAAC	$TAT(GT)_{22}$ ATG
D16S405	(F) CTCTGCTGCACCTGGC (R) ATGGGGACCATGAAGG	$(AC)_{25}$
D16S292	(F) TGTCAGGCCAGCCATGTTTT (R)CTTTGCACAAAAACAGTAGCTATCCAC	$(GCT)_7(GT)_{18}$

electrophoresis in 1% agarose gels and transferred to Hybond N+ nylon membrane (Amersham). Hybridisation of the probe to the membrane was performed at 42°C with a random-primer-labelled probe as per 2.3.4.2 (Promega) which was pre-reassociated with Cot-1 DNA in 5xSSC at 65°C (as per 2.3.4.4). To provide quantitative data, image analysis was performed using a PhosphorImager™ (Molecular Dynamics, Sunnyvale, CA) and scored as a ratio of the test probe (MRP#14 - deletion status unknown) to a control probe (LE12- known to be present in the genome in a two gene dosage and used to control for the amount of DNA in each track).

4.3.9 Statistical Analysis

For each patient in this study, "time to failure" was measured from the date of diagnosis until either death from any cause or to relapse of the leukaemia. Relapse was defined by the presence of leukaemic blasts on morphological examination of the bone marrow or the presence of inv(16) containing metaphases on cytogenetic examination of the bone marrow of the patient. Patients were omitted from analysis where the retention or deletion status of the *MRP* gene was uncertain. Distributions of time to failure were estimated by the method of Kaplan and Meier (1958). Since the number of patients was limited for comparing those with and without deletion of the *MRP* gene, exact p-values were calculated from the permutation distributions of Wilcoxon rank sum and log rank statistics as appropriate, using the commercially available StatXact Turbo program (Cytel Software Corporation, Cambridge, Massachusetts, USA).

4.4 Results

4.4.1 Making a unique probe from *MRP*#14

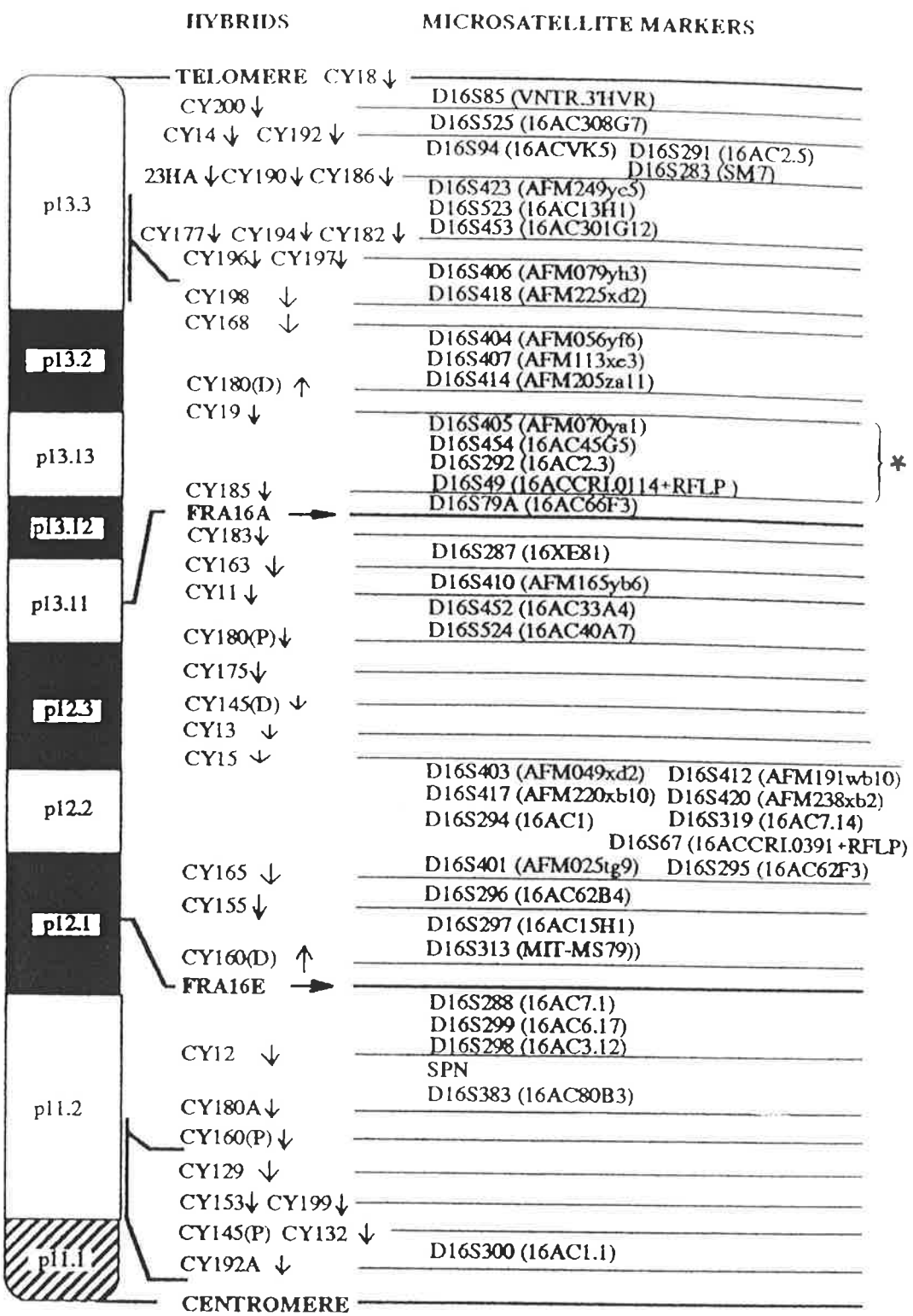
MRP#14 DNA (figure 4.1) was digested with a panel of restriction enzymes selected according to the restriction enzyme sequence analysis of the cDNA clone *MRP*#14 (Cole 1992). The digested DNA was Southern transferred to nylon membrane as per 2.3.5. This membrane was hybridised with ^{32}P labelled total human genomic DNA, washed and exposed to radiographic film overnight (2.3.4.2 and 2.3.6). Restriction fragments which did not contain abundant repeat sequences were isolated as per the method described in 2.3.10.5. These restriction fragments were cut out of a second agarose gel after electrophoretic separation of the restriction digests selected for unique bands. For confirmation of the uniqueness of this restriction fragment, the specific band was labelled and hybridised back to membranes containing restricted *MRP*#14 DNA and total human DNA. The resultant probe was an 812bp BglII-HindIII fragment from MRP#14.

4.4.2 Genetic mapping of *MRP* on chromosome 16

Oligonucleotide PCR primers MRP1F and MRP1R (4.3.5), designed from the 5' untranslated region of the cDNA sequence of *MRP*, amplified a 220 base pair product containing approximately 13 copies of the CCG triplet repeat. These primers were used to map the *MRP* gene by PCR to the interval defined by the somatic cell hybrids CY19 and CY185 (Figure 4.3) being positive for CY18, CY19, & CY198 and negative by PCR for CY185, CY11 and CY158. The latter somatic cell hybrids do not contain the 16p13.13 region encompassed by the interval between CY19 and CY185. This is the same region that contains the *MYH11*

Figure 4.3 Idiogram of chromosome 16p and somatic cell hybrid intervals

The somatic cell hybrid panel derived from chromosome 16 DNA and the hybrid intervals for the short arm of chromosome 16 are outlined. Arrows imply the inclusion of chromosomal DNA from the primary breakpoint for that hybrid. Microsatellite markers included in the intervals between hybrid panel members are listed in the side column. Hybrid interval CY19-CY185 encompassing the region denoted by 16p13.1 is marked.


HYBRIDS
MICROSATELLITE MARKERS
TELOMERE CY18↓
CY200↓
CY14↓ CY192↓
23HA↓CY190↓CY186↓
CY177↓ CY194↓CY182↓
CY196↓ CY197↓
CY198 ↓
CY168 ↓
CY180(D) ↑
CY19↓
CY185↓
FRA16A
CY183↓
CY163 ↓
CY11↓
CY180(P)↓
CY175↓
CY145(D) ↓
CY13 ↓
CY15 ↓
CY165 ↓
CY155↓
CY160(D) ↑
FRA16E
CY12 ↓
CY180A↓
CY160(P)↓
CY129 ↓
CY153↓ CY199↓
CY145(P) CY132 ↓
CY192A ↓
CENTROMERE
p13.3
p13.2
p13.13
p13.12
p13.11
p12.3
p12.2
p12.1
p11.2
p11.1
D16S85 (VNTR.3'HVR)
D16S525 (16AC308G7)
D16S94 (16ACVK5)
D16S291 (16AC2.5)
D16S283 (SM7)
D16S423 (AFM249yc5)
D16S523 (16AC13H1)
D16S453 (16AC301G12)
D16S406 (AFM079yh3)
D16S418 (AFM225xd2)
D16S404 (AFM056yf6)
D16S407 (AFM113xc3)
D16S414 (AFM205za11)
D16S405 (AFM070ya1)
D16S454 (16AC45G5)
D16S292 (16AC2.3)
D16S49 (16ACCRI.0114+RFLP)
D16S79A (16AC66F3)
D16S287 (16XE81)
D16S410 (AFM165yb6)
D16S452 (16AC33A4)
D16S524 (16AC40A7)
D16S403 (AFM049xd2)
D16S412 (AFM191wb10)
D16S417 (AFM220xb10)
D16S420 (AFM238xb2)
D16S294 (16AC1)
D16S319 (16AC7.14)
D16S67 (16ACCRI.0391+RFLP)
D16S401 (AFM025tg9)
D16S295 (16AC62F3)
D16S296 (16AC62B4)
D16S297 (16AC15H1)
D16S313 (MIT-MS79))
D16S288 (16AC7.1)
D16S299 (16AC6.17)
D16S298 (16AC3.12)
SPN
D16S383 (16AC80B3)
D16S300 (16AC1.1)
*

gene to which is localised the short arm breakpoint of the inversion chromosome 16. A number of the CEPH kindreds, representing normal individuals, were studied using these primers and showed the CCG repeat region to be polymorphic (figure 4.4) and inherited in a Mendelian manner (figure 4.5). The repeats shown in figure 4.4 show seven alleles designated A to G and figure 4.5 demonstrates the inheritance of the alleles within a CEPH kindred.

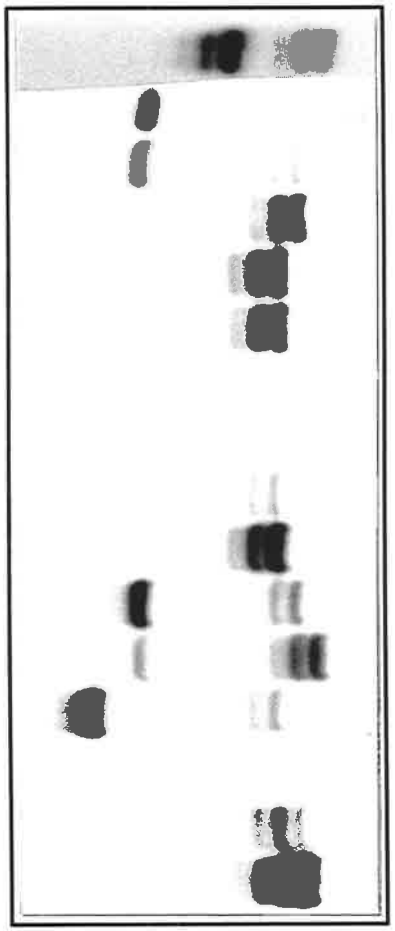
Amplification of the 5′ untranslated region of *MRP* demonstrated that in patient #1 there was virtually complete loss of one CCG containing allele in the bone marrow at diagnosis (figure 4.4). The reappearance of this allele in the bone marrow at clinical remission confirmed heterozygosity for this polymorphism as observed in the normal cells of patient #1. The differences seen in the allelic pattern suggested that a change from the normal heterozygous state occurred when the patient developed leukaemia at which time the allele pattern changed to a leukaemic hemizygous state, due to a deletion of one *MRP* allele.

The other patients studied showed either a homozygous pattern, or a heterozygous pattern for the GCC containing allele with apparent gene dosage differences (figure 4.4). Differences in band intensity of the amplified *MRP* gene alleles were suggestive of gene dosage differences, as predicted by subtle changes in the allelic pattern as shown in figure 4.4. When the gene dosage is equal for both alleles, the smaller allele preferentially amplifies which is seen with the amplification in Pt.#1(R). The reverse is seen when there is a predominance of the larger allele present in the DNA sample, as seen in Pt.#1, Pt.#11 and H69AR in which there is preferential amplification of the larger allele. However, the differences are not as marked in

Figure 4.4 PCR amplification of the CCG repeat of the 5′ untranslated region of *MRP*

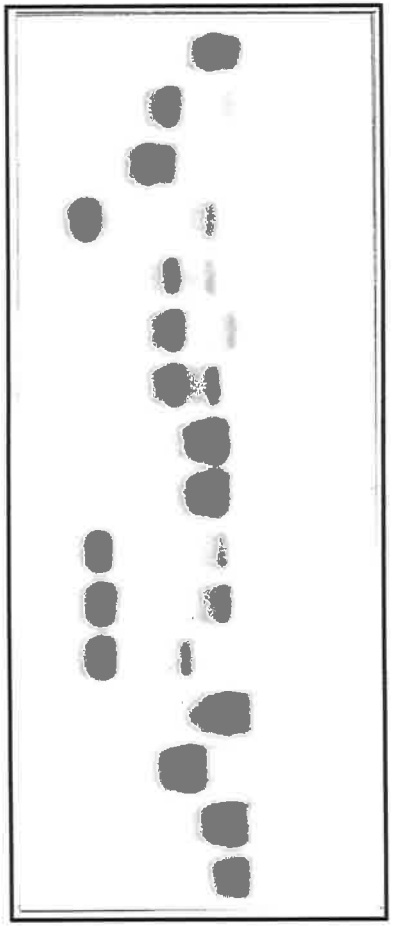
Figure 4.4 represents the autoradiographic features of the PCR amplification of the CCG repeat of the 5′ untranslated region of the MRP gene. The data in 4.4a is derived from a CEPH parent panel, numbers ending in 1 representing paternal derived DNA and numbers ending in 2 representing maternal derived DNA. These demonstrate the polymorphic nature of this repeat. The data in 4.4b represents PCR amplification of the CCG repeat from DNA from inv(16) leukaemia patients as listed in table 4.1. (R) denotes remission sample for that patient. CY10 is the somatic cell hybrid containing the inv(16) chromosome as its only human chromosome. H69 and H69AR are lung cancer cell lines. The lettering below both figures represents designated alleles for the CCG repeat: A-G. Patient #1 shows a change in the allelic pattern from leukaemic DNA sample to remission (R) DNA sample. Subtle changes in the allelic pattern (the larger allele being preferentially amplified instead of the smaller allele) for patient 11 and patient 1 were suggestive of unequal MRP gene dosage, but were not conclusive evidence of gene deletion.

B



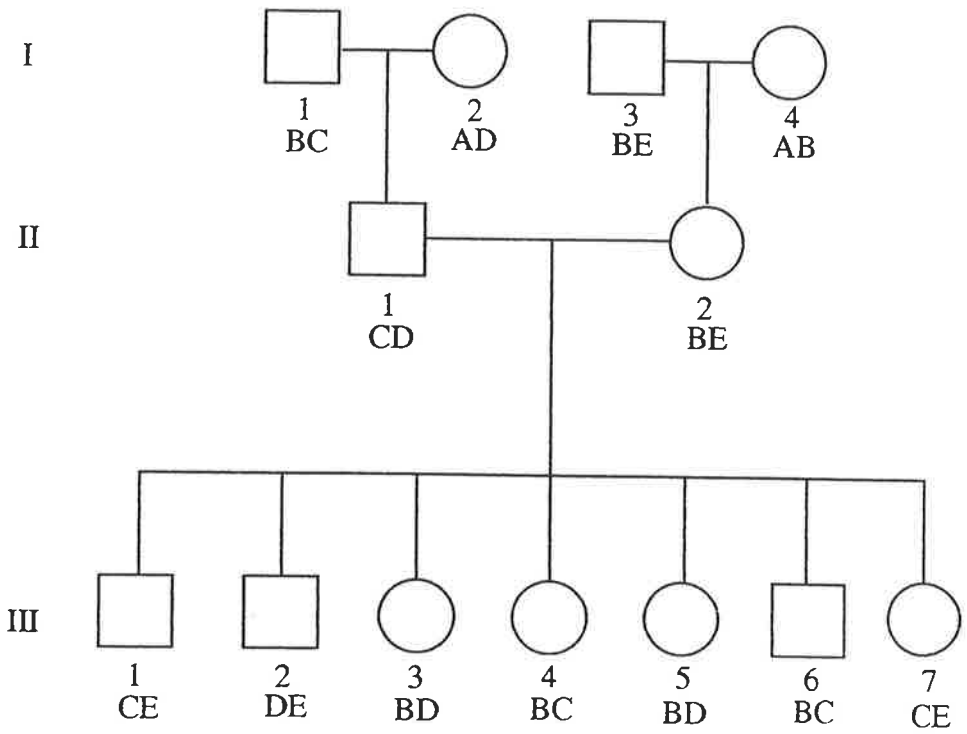
BE	Pt.3
AF	Pt.9
CF	Pt.1(R)
CF	Pt.1
D	Pt.2(R)
D	Pt.2
	Blank
	Cy10
D	Cy10
D	Pt.5
CF	Pt.8
BF	Pt.11
DG	Pt.6
	Blank
CD	H69
C	H69AR

A



AB	0201
CA	0202
CD	1201
BE	1202
BC	1701
AC	1702
BC	2101
B	2102
B	2301
BE	2302
BE	2801
CE	2802
AB	3501
C	3502
A	3701
A	3702

Figure 4.5 Pedigree of CEPH family for the CCG repeat found in *MRP*



The alleles for the CCG repeat of the 5′ untranslated region of the MRP gene were generated for a CEPH family and the results are demonstrated by the pedigree shown in this figure. Generation I are the CEPH grandparents, generation II the parents and generation III the children of the CEPH parents. Mendelian inheritance is shown for the PCR amplified alleles of the *MRP* gene.

Pt.#11 as for Pt.#1 and further corroborative evidence would need to be obtained, to be certain of deletion of one *MRP* gene in the leukaemic samples. Other methods were employed to detect the presence or absence of a single gene deletion in each of the remaining patients and the results are summarised in table 4.4. The other methods are detailed below in the following sections.

4.4.3 *In situ* hybridisation of *MRP* to inversion chromosome 16

Fluorescence *in situ* hybridisation (FISH) using the probe *MRP#14* was performed on metaphase spreads derived from bone marrow obtained at diagnosis from patients #1,#2,#3,#4,#7,#10,#12,#13-22 and results are summarised in tables 4.4 and 4.1. For each patient, a minimum of 12 metaphases were examined for fluorescent signal, except in patient #10 where only 4 metaphases were satisfactory for analysis. In patients #1,#2,#3,#12,#13,#14 and #20 no signal was seen on any of the inverted 16 chromosomes, while the normal chromosome 16 in the same cell, showed a fluorescent signal (figure 4.6). Analysis of the bone marrow of patient #10 revealed a mosaic pattern (table 1), both in the presence of the inversion chromosome 16 and the absence of the MRP#14 probe signal from the inverted chromosome 16. Since only a limited number of metaphases could be examined, it was impossible to assess the presence or absence of a deletion in this patient by this method. Fluorescent signal was seen on both the normal and inverted chromosomes 16, in patients #4,#15-#19, #21 and #22 suggesting the presence of two intact *MRP* genes in these patients. In these patients, the fluorescent signal was present on the short arm of the normal chromosome 16 and on the arm not containing the heterochromatin in the inv(16) chromosome.

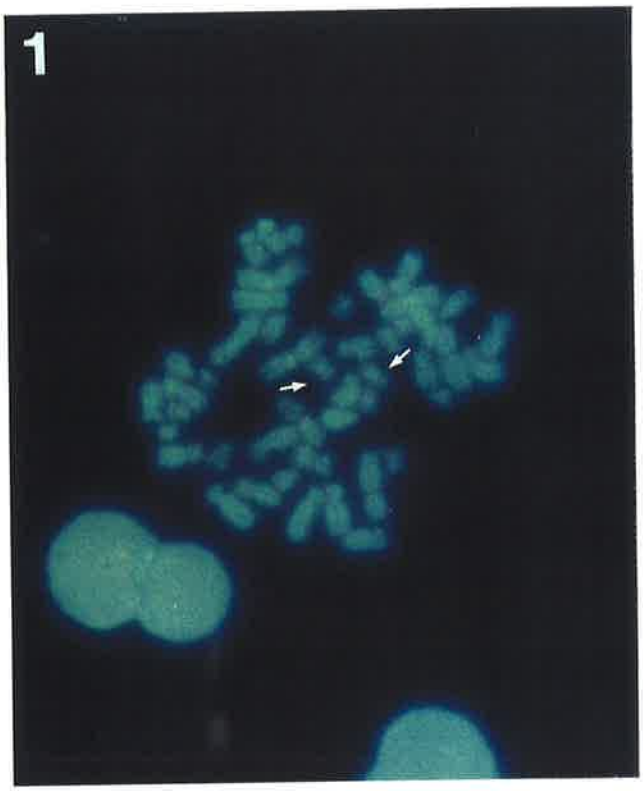
Table 4.4 Molecular detection of the *MRP* gene deletion and related clinical progress

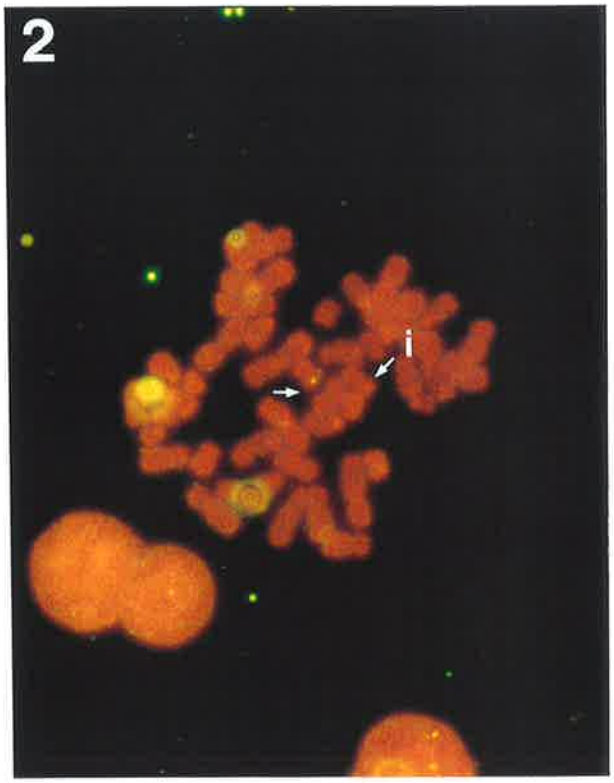
Pt.	FISH (MRP#14)	*D16S405*	Southern (MRP#14)	Time to Failure[f] (Weeks)	Treatment
1	Deleted	Hom-Het	Reduced	37 A[c]/CCR[d]	AraC-DNR[g]
2	Deleted	Hom-Het	Reduced	111 A/CCR	AraC-DNR[g]
3	Deleted	NA	NA	200 A/CCR	AraC-DNR[g]
4	Not Deleted	Het-Het	Not deleted	51 A/prog.	AraC-DNR[g]
5	NA[a]	Het-NA	Not deleted	65 A/prog.	AraC-DNR[h]
6	NA	Hom-NA	Not deleted	26 D/prog.	AraC-DNR[h]
7	Not Deleted	Het-NA	Not deleted	1.5 D/hrrg[e]	AraC-DNR[i]
8	NA	Het-NA	Uncertain	172 A/CCR	AraC[j]
9	NA	Hom-NA	Not deleted	27 D/prog.	AraC[k]
10	Mosaic	Het-NA	Not deleted	15 A/CCR	AraC-DNR[g]
11	NA	Het-NA	NA	3 D/sepsis	AraC-DNR[g]
12	Deleted	NA	NA	44 A/CCR	AraC-DNR[g]
13.	Deleted	NA	NA	154 D/prog.	AraC-DNR[g]

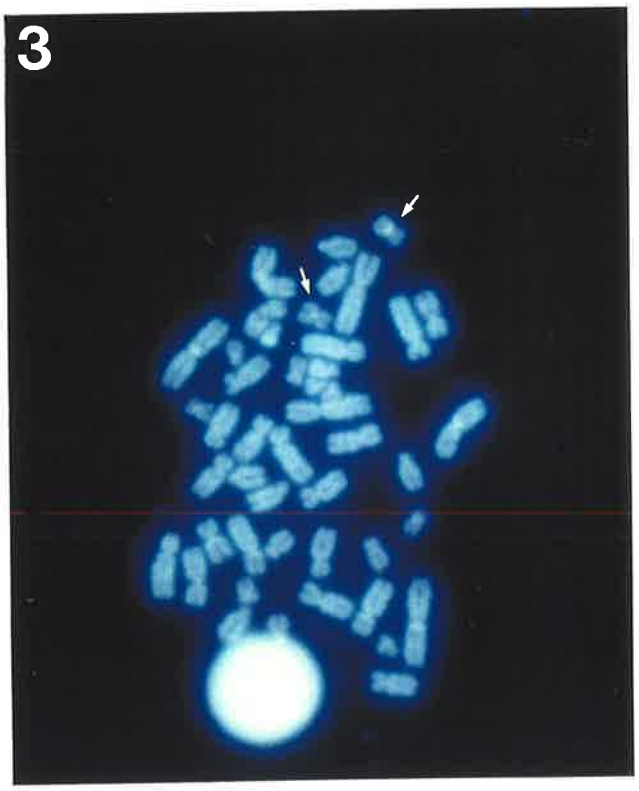
a NA: sample not available for analysis
b hom(homozygous), het(heterozygous) for locus D16S405 at time of diagnosis followed by remission (diagnosis-remission)
c A(alive); D(deceased)
d CCR(continued complete remission); Prog(progression of disease)
e haemorrhage
f From date of diagnosis to death or relapse, status at last follow-up
g AraC-DNR: cytosine arabinoside (200mg/M2/d days1-7) by continuous infusion (CI) and daunorubicin (50mg/m2 days 1,3,5)
h AraC-DNR: AraC (200mg/M2/d days1-7) CI, DNR 45mg/M2/d days 5-7
i AraC-DNR: AraC 2gm/M2 every 12 hrs x 12 (days 1-6), DNR 45mg/M2/d days 7-9
j AraC 2gm/M2 every 12 hrs x 12 (days 1-6)
k AraC 3gm/M2 every 12 hrs x 12 (days 1-6)

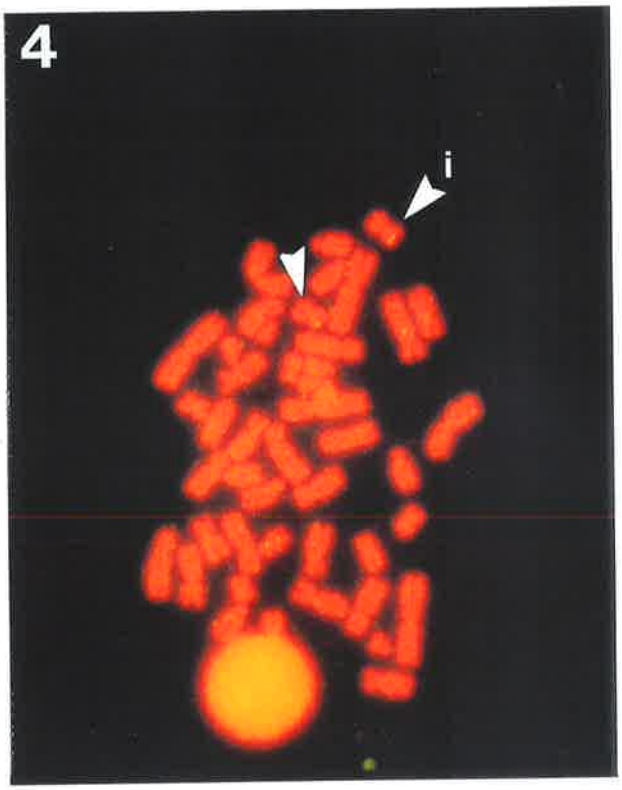
Figure 4.6 Analysis of metaphase chromosomes by FISH using *MRP#14*

Metaphase spreads of leukaemic cells containing an inverted chromosome 16 and a normal chromosome 16. A DAPI stain has been included to allow identification of the two chromosomes 16 **(#1,#3)**. The inverted chromosomes 16 are labelled **(i)**. The FITC labelled MRP#14 probe has been hybridised to the chromosomes using FISH techniques and is shown to be present on the normal chromosome 16 only (arrowed left) in **#2** and on both chromosomes in **#4**. No signal is seen on the inverted chromosome 16 (arrowed, **#2**) in which a deletion of the MRP gene has occurred.


1


2
i


3


4
i

The data presented in this section confirmed the presence of the *MRP* gene on the short arm of chromosome 16. The position of *MRP* on the inv(16) chromosomes which do not have a deletion established that the *MRP* gene is proximal to the breakpoint and confirmed the data of Liu (1993) in demonstrating that the deletion is proximal to the short arm breakpoint. It demonstrated for the first time that the deletion involved in all cases studied, the multidrug resistance gene *MRP* (figure 4.6).

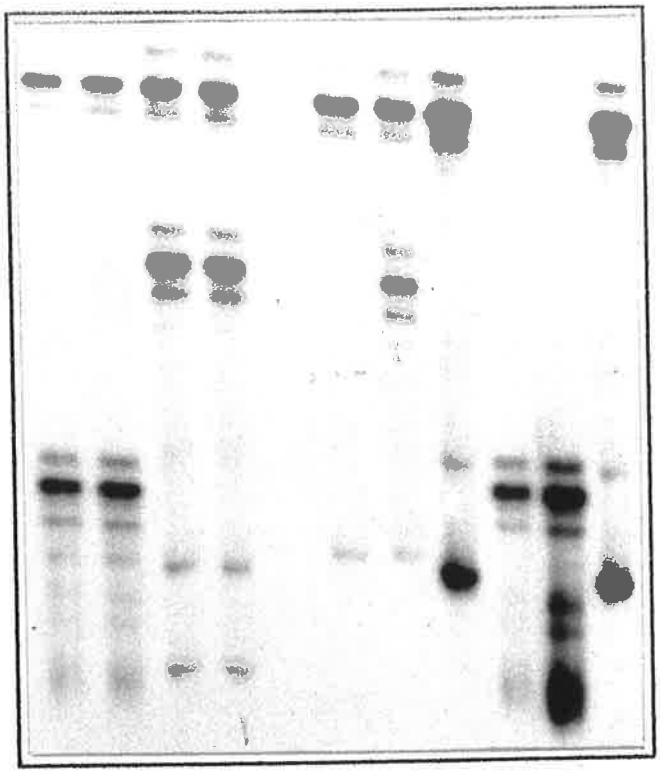
4.4.4 Microsatellite marker analysis: evidence for deletion

Figure 4.7 shows PCR products from the microsatellite markers *D16S49* and *D16S405*. Results for *D16S292* and *D16S454* were the same as those for *D16S49* and are not shown. Lanes 1/2, 3/4 (*D16S49*), 12/13 and 14/15 (*D16S405*), represent amplification from DNA extracted from bone marrow of patients at remission and diagnosis respectively. The samples listed in this figure are from patients as listed in table 4.2. The alleles amplified by *D16S49* for patients #1 and #2, were heterozygous and identical in DNA extracted from cells at times of remission and to that obtained from DNA extracted from the leukaemic cells from that patient. This suggested that the locus for *D16S49* is not involved in a deletion associated with the inv(16). However, for both patients, there was loss of one allele in the leukaemic bone marrow at diagnosis for *D16S405*, as compared with the heterozygous pattern for the allele in the normal bone marrow at remission. Patient #10 showed a heterozygous pattern both at presentation and relapse consistent with no deletion of this locus in the inv(16) chromosome (table 4.4). This result would be expected, considering the mosaic karyotype of the leukaemic cells from that patient (see 4.4.3).

Figure 4.7 Analysis of microsatellite markers from region CY19-CY185

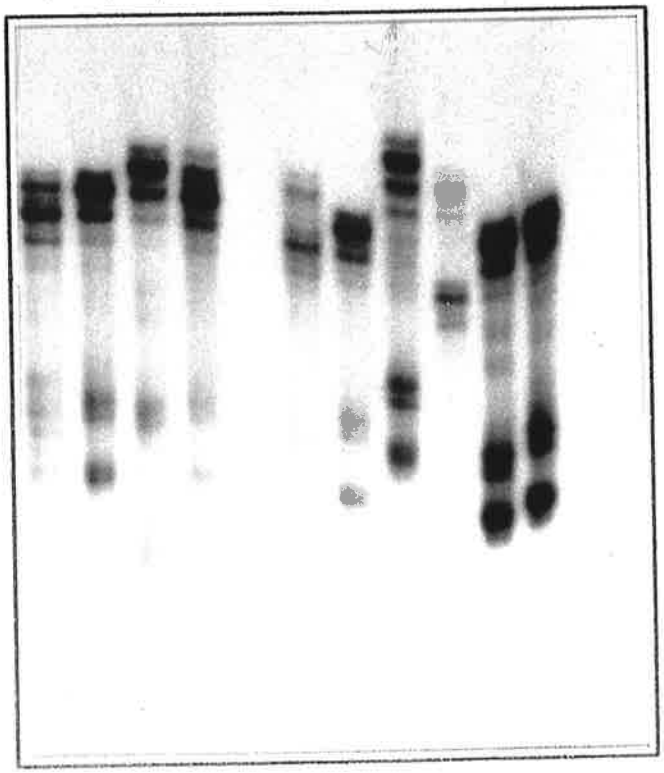
^{32}P labelled PCR products of microsatellite regions (D16S49 and D16S405), amplified from total human DNA from patients with inversion chromosome 16 leukaemia, at time of remission (R) and/or diagnosis (L). Products have been separated by gel electrophoresis on a 4% polyacrylamide gel. Lanes (1,2)&(12,13) = patient 1; (3,4)&(14,15) = patient 2; (6&17) = patient 8; (7&18) = patient 6; (8&10) = patient 9 (9&20) = patient 5. Lanes (10&21) = patient 7/CY10 and (35&46) = Cy18 represent single allele controls. At the locus D16S405, patients 1 & 2 show a change in allele pattern from hemizygous (due to a single gene deletion), when leukaemic cells are analysed at the time of diagnosis, to heterozygous at the time of remission. At locus D16S49, no change in allele pattern is observed.

A



Lanes	1	2	3	4	5	6	7	8	9	10	11
Status	R	L	R	L		L	L	L	L	L	
Patient no.	1	1	2	2		8	6	9	5	7	Cy18
Lanes	12	13	14	15	16	17	18	19	20	21	22

B



In those patients where both diagnosis and remission samples were not available (table 4.4), leukaemic patterns only could be assessed. Patients #5, #8 and #11 showed heterozygous patterns for locus *D16S405* in the leukaemic state, again suggesting no deletion had occurred involving this locus. Patients #6 and #9 were homozygous for the *D16S405* locus. However, without remission samples, it was impossible to differentiate homozygosity from hemizygosity and so no conclusions could be drawn from analysis of the samples from these two patients.

These data are consistent with the locus *D16S405* being deleted along with the MRP gene in some cases of the inv(16) and therefore positions it centromeric to the primary breakpoint within the *MYH11* gene. However, it did not establish an order for *MRP*, *D16S405* and *MYH11*. It was not possible to establish whether *D16S405* may be part of the genomic sequence for MRP as no cloned genomic DNA for *MRP* was available at that time. However, YAC clones and a cosmid contig (Dauwerse 1993) were available in the region of the short arm breakpoint and were obtained from Dr Martijn Breuning.

4.4.5 Estimation of deletion size and distance of *MRP* from the primary breakpoint

The sizes of the deletions associated with the inv(16) have been estimated by pulsed field electrophoresis to vary in size from 150-360kb (Marlton 1995). Using the cosmid contig mentioned in 4.3.3.2, the minimum distance between *MRP* and *MYH11* was estimated. The most proximal cosmid of this contig, cosmid zit 79, was found by PCR and southern analysis using oligonucleotides from the 5' region of MRP and a unique 218 base pair *MRP*#7

restriction fragment (as described in 4.3.3.1), to contain part of the 5' region of MRP, being positive for these specific probes (figure 4.8)

These cosmids established the approximate distance separating the *MRP* gene from the primary breakpoint cluster region and placed the 5' end of the MRP gene including the promoter region, approximately 150kb from the cluster of breakpoints in the MYH11 gene. This potentially places the 5' ends of *MYH11* and *MRP* in close proximity and implies that the *MRP* gene is transcribed in a telomeric to centromeric direction.

Cosmid zit14 of this contig was found to be positive by PCR for the microsatellite marker D16S405, already known to be positioned in the same interval as *MRP* and *MYH11* and centromeric to *MYH11*. These data established the order of the three probes as: centromere - *MRP, D16S405* and *MYH11* - telomere as per figure 4.9. These data confirm the conclusions drawn from the microsatellite marker analysis of patient DNA namely that *D16S405* is included in the region deleted from the inversion chromosome and that the deletion is likely to extend proximally from the primary breakpoint of each particular patient.

Using the PCR primers constructed within the promoter region of *MRP* (MRP1F and 1R), YAC libraries and cosmids already mapped to the region by Dr Norman Doggett at the Los Alamos National Laboratories, were screened. The data resulting from this is shown in figure 4.9. When the cosmid clones received from Los Alamos were checked by Southern analysis using MRP#14 as a probe, there were no true positive cosmids received and unfortunately, no mega-YAC contained all three probes of interest namely *MRP, MYH11* and the microsatellite

Figure 4.8 Restriction map of cosmid zit 79

Following Southern transfer of cosmid DNA, T7 & T3 oligonucleotides (end probes) and a 218bp restriction fragment of MRP#7 were hybridised to the membrane. A 7.5kb HindIII/BglII restriction fragment of zit 79 was found to be positive for MRP#7. Comparison of cosmid and human restriction fragments hybridised to the MRP#7 fragment, revealed a deletion of uncertain size at the T3 end of the linearised cosmid.

Restriction map of cosmid zit 79

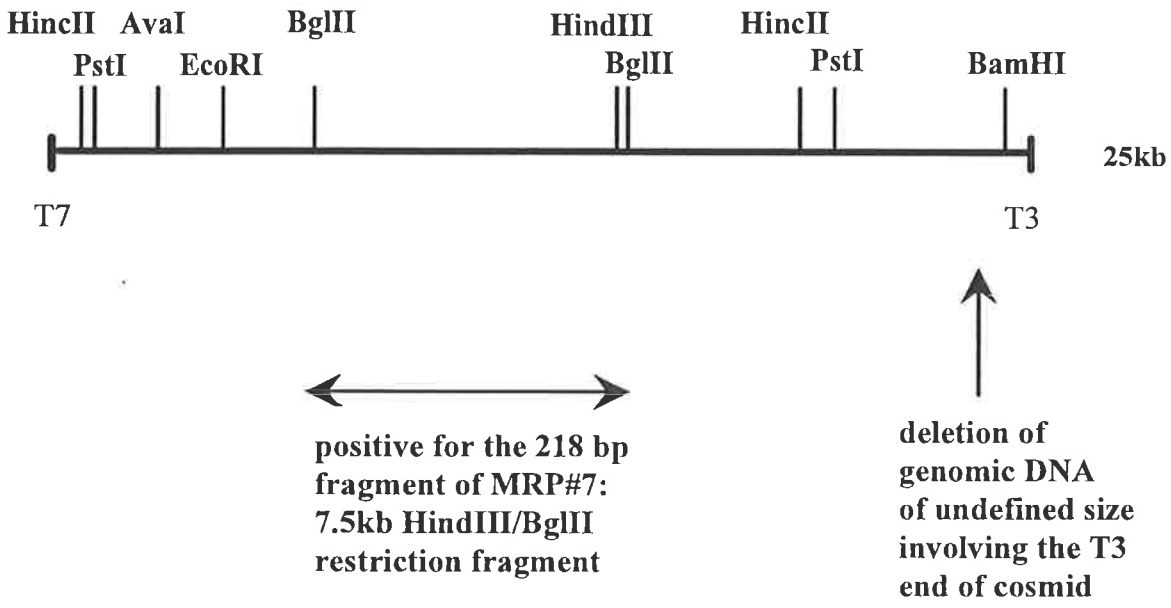
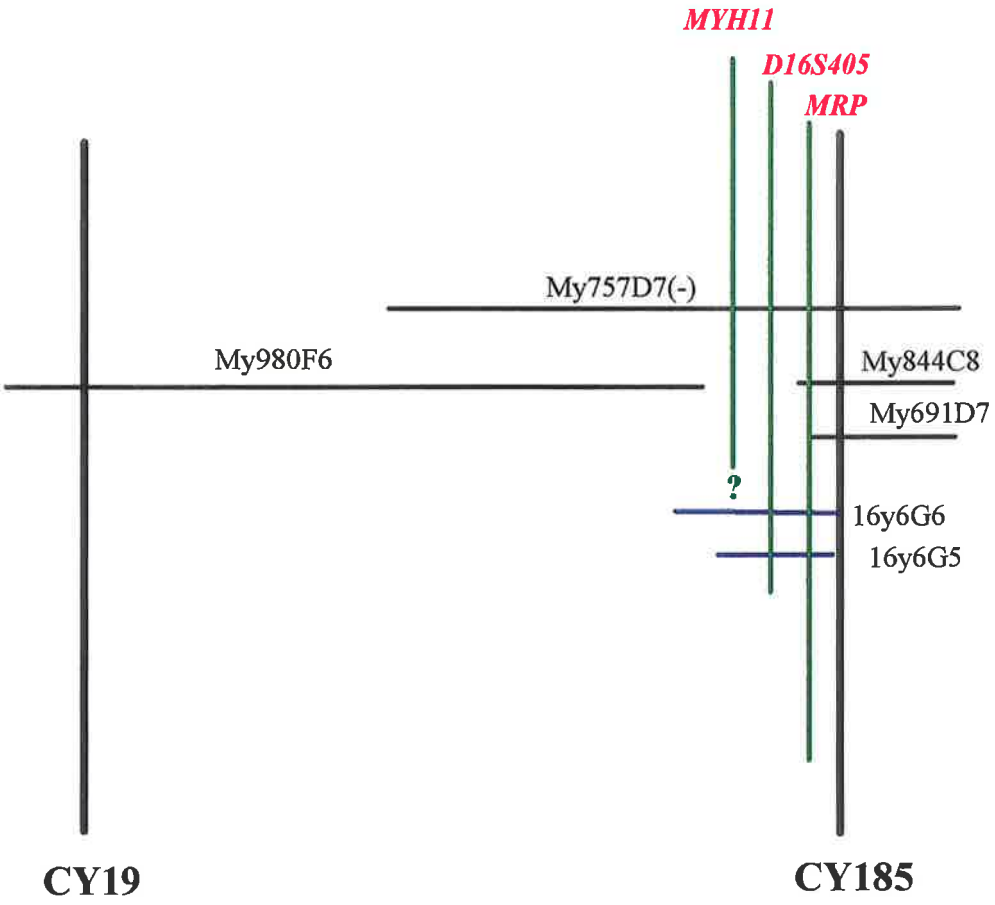


Figure 4.9 Gene and YAC map adjacent the inv(16) p arm breakpoint

The CY19-CY185 interval is depicted in the idiogram. The figure is not accurately drawn to scale. The relative positions of *MYH11*, *MRP* and the microsatellite marker *D16S405* are shown by vertical lines. The mega-YAC 757D(-) is positive for *MYH11* and the microsatellite marker but not for MRP. The minus sign implies a deletion has occurred in the making of the YAC. My844C8 and My691D7 are positive for *MRP*. 16y6G6 and 16y6G5 are both positive for *MRP* but negative for *D16S405*.

Gene and YAC Map adjacent the inv(16) p arm Breakpoint



marker *D16S405*. This was due to a series of deletions within the mega-yacs belonging to the CEPH chromosome 16 mega-yac library, encompassing the regions of human DNA represented by *MYH11*, *MRP* and *D16S405*.

4.4.6 Gene dosage studies

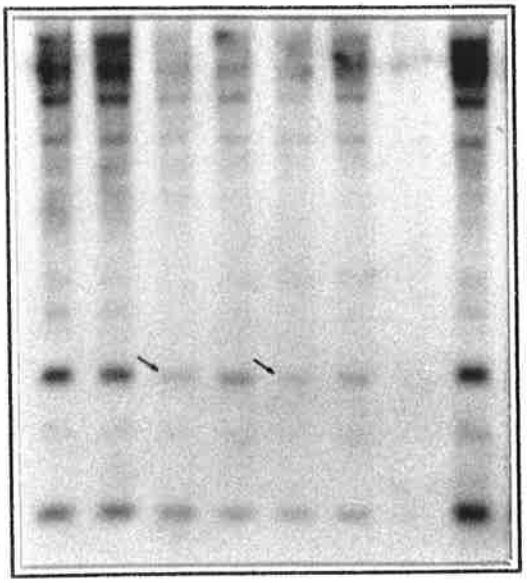
Where DNA was available, BamHI restriction digests of DNA from patient bone marrow samples were probed simultaneously with *MRP#14* and LE12, a control chromosome 16 probe. The autoradiograph is shown in figure 4.10. Quantitation of the 1.8kb and 1.2kb bands representing hybridisation of the probes *MRP#14* and LE12 respectively, revealed a lower signal ratio (*MRP*#14/LE12) for the leukaemic samples (arrowed patients #1 & #2) when compared with both their remission bone marrow samples which contained normal karyotypes, and normal controls. This is consistent with a lower gene dosage in the leukaemic state representing a deletion involving *MRP#14*. Patients #5,#6,#7,#9 & #10 showed ratios equivalent to the gene dosage of controls on the same filter. Patient #8 showed equivocal results on repeat hybridisations making reliable assessment difficult. This patient also showed a high percentage of normal lymphocytes present in the bone marrow and peripheral blood at diagnosis when compared with other patients.

4.4.7 Survival analysis of patient data: Correlation with gene dosage

Data regarding times to failure are listed in table 4.4 for thirteen patients. As previously stated, clinical data was not available on the remainder of the patients. Six patients were alive in continuous complete remission between 37 and 200 weeks after diagnosis, five achieved complete remissions but have relapsed, and two died within 3 weeks of diagnosis.

Figure 4.10 Analysis of *MRP* gene dosage by Southern

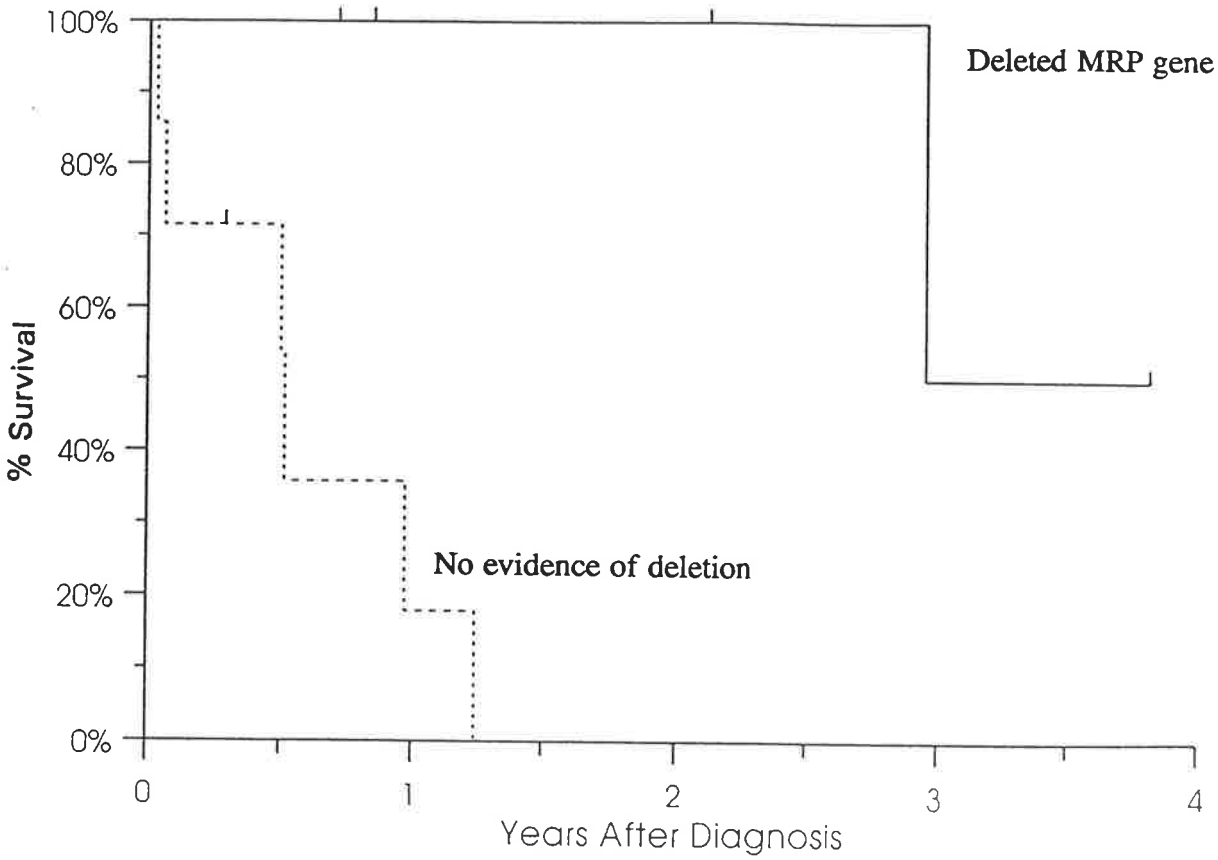
Southern analysis of BamHI digests of total human DNA, extracted from patients with inversion chromosome 16 containing leukaemia, at time of diagnosis and at remission. Digests have been probed simultaneously with MRP#14 and LE12 randomly labelled, radioactive DNA probes. Quantitative analyses of the bands, to assess gene dosage, are represented as ratios of the intensities of the 1.8kb band representing the MRP#14 probe and the 1.2kb band representing the control probe, LE12. Relative intensities of the 1.8kb bands in the arrowed lanes are reduced and represent reduced *MRP* gene dosage.


1.8kb
1.2kb
MRP#14
LE12
a b c d e f g h
Patient no.
1 2 10
Ratio of Intensity
1.1 1.1 0.7 1.3 0.8 1.1 0.9 0.9
Leukaemic
Remission

Kaplan Meier estimates of the distributions of time to failure for patients with and without deletion of the *MRP* gene are shown in figure 4.11. Patient #8 was omitted from this analysis since his *MRP* deletion status was indeterminate. Times to failure were significantly longer for patients with deletion of the *MRP* gene (two-tailed p = 0.003 by the log rank test). The five patients with deletion of the *MRP* gene had a median age of 25 years, compared to 41 years for the seven without deletion of the *MRP* gene. Although this difference was not statistically significant (p = 0.16 by the Wilcoxon rank sum test), its magnitude suggested that the apparent difference of times to failure might be at least partly a consequence of age difference. However when the log rank analysis of time to failure was stratified by age (<35 vs. 35+), the effect of *MRP* gene deletion remained statistically significant (p = 0.007). The inclusion of patient #11 among those without deletion of the *MRP* gene might be questioned, however if she is omitted, the results are not substantially changed (log rank p = 0.004 and 0.013 for comparison of times to failure based on unstratified and stratified tests, respectively).

Also, the results were not primarily due to the occurrence of the two early deaths (patients #7 & #11) in the group without deletion of the *MRP* gene: with those two patients omitted the unstratified and stratified log rank p-values were 0.008 and 0.02, respectively. The only other karyotypic difference is the additional chromosomes (+8 and +22) in patients #6, #7 & #9. Additional cytogenetic abnormalities have not been shown to affect prognosis in a larger study (Campbell 1991).

Figure 4.11 Survival analysis for leukaemic patients by *MRP* gene deletion



Kaplan Meier estimates of distributions of time from diagnosis to failure (death or relapse from complete remission) among patients with inversion chromosome 16 AML. Comparison of 5 patients with an *MRP* gene deletion (solid line) and seven patients without an *MRP* gene deletion (dashed line), (P=0.007). Patient 8 is omitted. Tick marks indicate censored observations for five patients last known to be alive in CCR.

4.5 Conclusions and Discussion

The results of this study indicate that *MRP* maps to the band 16p13.13. The *MRP* gene is deleted in a subgroup of patients in which an interstitial deletion of the chromosome 16 occurs in association with the inv(16) event. This event has been correlated with an improved duration of first remission in a group of thirteen patients, suggesting that the deletion of *MRP* may affect the biological response of the leukaemia to chemotherapy. From the mapping of the 5′ end of *MRP* to the cosmid zit79, and the absence of any other cosmids from this contig distal to zit79 being positive for *MRP*, it can be concluded that the *MRP* gene is orientated 5′ to 3′ from telomere to centromere. This implies that the *MRP* gene is transcribed in the opposite direction to MYH11 (Liu 1993). The estimated distance from the 5′ region of MRP to the *MYH11* breakpoint cluster region is 150kb. It is not known whether there are any intervening genes between *MRP* and *MYH11*, however if there are none, this would imply that the 5′ regions of the two genes may be juxtaposed (figure 4.2).

In this study MRP was deleted in 7 out of 22 patients (31.8%) with inv(16) leukaemia. The region of the gene included in probe MRP#14 encompasses the translational initiation site and almost 4 kb out of the 6.5kb total coding sequence. Consequently, it is highly probable that the deletions detected with this probe will completely inactivate the *MRP* gene on that chromosome. A deletion of the short arm of chromosome 16 in association with the short arm breakpoint of the inv(16)(p13q22), has been previously documented (Liu 1993, Dauwerse 1993). The data represented in this chapter further define the extent of the deletion and show that the *MRP* gene is included in this deletion. It is proposed that deletion of the *MRP* gene can begin to explain the prognostic significance currently attached to this

chromosomal abnormality. Times to failure for the group in which the *MRP* gene is deleted are significantly longer compared to the group with no demonstrable deletion (p=0.007 with adjustment for age). The heterogeneity of the clinical course of patients within the inv(16) leukaemia subgroup of acute leukaemias, has been previously documented but never before correlated with genetic differences (Ohyashiki 1988, Campbell 1991).

In this study, patient #8 appears clinically to belong to the group containing the deletion but is heterozygous for the microsatellite marker (*D16S405*). This is likely to be due to the high percentage of lymphocytes present in the bone marrow and peripheral blood at diagnosis when compared with other patients. It is also possible that a small but critical deletion may be present in this patient which may not involve the *D16S405* locus. It is likely that the size of the deletion varies from patient to patient (Marlton 1995). Other genes may be involved in the deletion and be of further significance to the behaviour of inversion 16 leukaemias.

The difference in time to failure of the two groups of patients in this study is significant and appears comparable or greater than differences shown to date, in clinical trials comparing treatment regimens, age or karyotypic abnormalities. This difference is relevant in terms of the timing of treatment options such as bone marrow transplantation, offered to these patients. The additional cytogenetic abnormalities seen in the "non-deleted" group (+8,+22) have been previously documented in a study of 26 patients with inv(16) AML or myelodysplasia (Campbell 1991). No statistically significant difference in survival was found between the inv(16) only group (n=12) and the additional chromosomal abnormality group (n=11), the median duration of survival for the whole group being approximately 12 months. However

there is some data which suggests trisomy 8 may be a poor prognostic indicator in acute leukaemia (Pederson-Bjergaard 1990, Walker 1994) although the evidence for this is not as strong as the evidence for the favourable prognosis of the inv(16) group as a whole. No specific information with regard to treatment regimen was elaborated on in Campbell's study (1991), however it is so far the most comprehensive study of the survival of inv(16) leukaemic patients.

A larger study involving 48 inv(16) leukaemic patients from the MD Anderson Cancer Center, Houston, Texas, USA, did not find a significant correlation between the presence of a short arm deletion and prognosis (Marlton 1995). The probes used to determine the presence of a deletion were 4kb and 10kb from the breakpoint cluster region, far short of the 150kb distance of *MRP* from the breakpoint cluster region, and an *MRP* specific probe was not used. It is possible however that due to the proximity of the primary short arm breakpoint in *MYH11* and *MRP*, that a functional disruption of the *MRP* gene may occur due to the molecular rearrangement of the inv(16) alone, without a deletion. This may be due to transcriptional regulatory elements for *MRP* being situated a considerable distance upstream of the transcriptional start site of *MRP* as is the case with the globin genes and the locus control region (LCR) (Bresnick 1994). The LCR is situated up to 50kb from the globin genes transcriptionally regulated by the LCR. Quantitative measurements of *MRP* mRNA and protein are required to investigate the differences between the group with a deletion of the *MRP* gene, and those without a deletion. Further information regarding the promoter region of *MRP* and the factors involved in its regulation are also required to fully understand the effects that the inv(16) event may have on *MRP* expression.

While it is inferred that the association between reduction to hemizygosity and improved prognosis is attributable to increased drug sensitivity of the leukaemic cell population, as the result of an *MRP* gene dosage effect, the expression and role of the *MRP* gene in haematopoietic stem cells is yet to be determined. Early reports have not shown marked differences in the expression of *MRP* between drug resistant and sensitive leukaemic populations (Hart 1993, Abbaszzadegan 1994). However, later studies are showing differences in the expression of *MRP* in leukaemic blasts, of the order of 1.7 fold increase over the expression of *MRP* in a drug sensitive cell line, which correlates with an increase in anthracycline transport from the cell (Ross 1995). Normal peripheral blood mononuclear cells contain readily detectable levels of *MRP* mRNA (Cole 1992, Zaman 1993) and drug resistance associated with HL60/ADR cells (an acute promyelocytic leukaemia derived cell line, subsequently exposed to cytotoxic agents to induce drug resistance) correlates with the overexpression of *MRP* mRNA and protein (Krishnamachary 1994). Thus loss of one *MRP* allele in leukaemic cells may serve to increase their sensitivity to daunorubicin relative to both normal haematopoietic stem cells and to leukaemic stem cells of other AML subtypes.

The underlying reason for chromosomal breakage and deletion is in generally poorly understood. One hypothesis for a potential mechanism may involve fragile sites (Yu 1991) due to the expansion of perfect repeat elements within genes, such as $p(CCG)_n$ repeats (Kremer 1991) and GC rich sequences of DNA (Jones 1995). There are a number of examples where repeat sequences are situated close to or within genes involved in chromosomal rearrangement. Both the *CBFB* (core binding factor β) gene involved in the long arm

breakpoint of the inv(16) leukaemias and the *MRP* gene have CCG repeats in their 5′ untranslated regions. Other genes include the *BCR* gene involved in chronic myeloid leukaemia [t(9;22) *BCR/ABL* rearrangement] and also the MLL (mixed lineage leukaemia) gene situated at 11q23 which is involved in a number of leukaemic and lymphomatous chromosomal rearrangements [eg. t(4;11), t(11;19), t(6;11)]. The breakpoint cluster region on chromosome 17 involved in the development of acute promyelocytic leukaemia involves the first intron of the retinoic acid receptor alpha gene which contains two CpG rich islands (Borrow 1990). In a recent report by Jones (1995), the FRA11B fragile site was localised to the $p(CCG)_n$ repeat of the CBL2 proto-oncogene. A proportion of patients in whom a genetic disorder due to a constitutional deletion of the long arm of one chromosome 11 had been diagnosed, were shown to inherit a chromosome carrying a CBL2 $p(CCG)_n$ expansion. It was then demonstrated that the 11q- breakpoint consistently mapped within a 20kb region of the chromosome 11 containing the triplet repeat expansion. The conclusion drawn from this being that a relationship existed between the triplet repeat and the tendency for deletion.

It is noteworthy that both the *CBFB* and the *MRP* gene have CCG repeats in their 5′ untranslated regions. In this study the 5′ untranslated CCG repeat was used as an inherited polymorphic allele to examine the possibility of deletion of the MRP gene. It was also noted that there appeared to be no significant size difference between the alleles of the CEPH kindred examined and the patients in whom an inversion of one chromosome 16 had occurred (figure 4.4). This implied that no obvious expansion of the CCG repeat was detectable by this method which may have predisposed the inv(16) leukaemic population to the chromosomal rearrangement. Understanding the mechanisms for chromosomal breakage and

the reasons for the involvement of particular regions of the genome in such mechanisms, would be of great significance to many areas of medicine.

Expression of *MRP* in inv(16) leukaemia

Chapter 5

5.1 Introduction

The results of chapter four of this thesis suggested that the deletion of the *MRP* gene in association with the short arm breakpoint of the inv(16) was associated with a longer time to failure of first remission of the associated leukaemia. To examine the possible cause for this association it was necessary to assess the level of expression of the *MRP* gene in inv(16) leukaemic samples. There is evidence to suggest that the level of expression of the *MRP* gene correlates with the degree of drug resistance manifested by that cell. This was demonstrated in HeLa cells (human epithelial, cervical carcinoma cell line) transfected with *MRP* cDNA, which then expressed vector encoded *MRP* mRNA and a 190kDA encoded protein. Analysis of the transfected cells showed a 5-15 fold increase in doxorubicin and daunorubicin resistance as well as a similar level of cross resistance with vincristine and VP16, commensurate with the level of vector encoded *MRP* mRNA transcribed (Grant 1994, Cole 1994). In addition, recent analysis of relapsed AML patients detected a 2 fold increase in *MRP* mRNA at relapse following induction chemotherapy, compared with levels at diagnosis, prior to the exposure to cytotoxic agents (Schneider 1995). Ross (1996) demonstrated in acute myeloid leukaemic blast cells, that a 1.7 fold increase in *MRP* expression compared with a drug sensitive cell line, correlated with a decrease in anthracycline accumulation at the cellular level. Therefore it is suggested that if there is a deletion of one *MRP* allele in inv(16) leukaemias, this could result in a significant reduction in cellular multidrug resistance and contribute to the chemosensitivity associated with the inv(16) leukaemias.

The assertion that the deletion of one allele of *MRP* would make a significant difference to the expression of drug resistance depends upon the level of expression of *MRP* within normal haematopoietic precursors at a similar stage of differentiation as the leukaemic blast and the contribution that this gene makes to the overall level of drug resistance within that cell type. For the purpose of matched controls, the immunophenotype analysis of the leukaemic cell needs to be considered in order that similar cell types to the leukaemic cell type be used in the analysis. This is difficult since it is impossible to perfectly match the phenotype of a leukaemic cell with a normal haematopoietic precursor, as leukaemic cells frequently co-express surface molecules which represent different lineages and different stages of differentiation. For example, in the case of the inv(16) leukaemias, the aberrant expression of CD2 has been documented, normally only expressed on thymocytes, T cells and some NK cells (Adriaanson 1993). However, comparisons between the leukaemic cell type and the normal control can be made using a best fit analogy and utilising a range of different "control" cell types. The cell types chosen in this analysis were flow sorted CD34+ cells, monocytes and non-flow-sorted bone marrow cells. In this chapter, the method of RT-PCR was chosen to analyse the leukaemic cells for the expression of *MRP,* and *MRP* deletion status was assessed using interphase FISH and Southern technology.

5.2 Aims

It was hypothesised that the level of *MRP* expression in the inv(16) leukaemias, would correlate with the number of intact copies of *MRP* genes present in that particular leukaemic sample. In addition, this reduced level of expression of *MRP* mRNA would be associated

with an improved clinical outcome for the patient. To assess the expression of the *MRP* gene in inv(16) leukaemias, a semi-quantitative RT-PCR technique was chosen. *MRP* expression in leukaemic samples was compared with normal haematopoietic precursors using the same method. A small sample of other leukaemic cell types and the leukaemic cell line U937, were also analysed to allow comparisons with the expression of *MRP* in other leukaemic cell types cited in the literature. The level of expression of *MRP* was then to be compared with the *MRP* gene deletion status for each patient and correlated to the clinical outcome where this information was available.

5.3 Materials and Methods (specific to this chapter)

5.3.1 Collection of Samples

A total of 29 patients with a histopathological diagnosis of AML and a bone marrow karyotype which included either the inv(16)(p13q22.1), t(16;16)(p13;q22.1), or del(16)(q22) were retrospectively studied. Cryopreserved bone marrow aspirates collected at the time of diagnosis were morphologically classified to confirm the diagnosis of acute myeloid leukaemia according to FAB classification criteria, and were karyotyped by routine procedures to confirm the presence of the rearranged chromosome 16. Table 5.1 provides patient clinical details, karyotypes and white cell counts, at presentation where known. Patients #1 - #16 and #19 - #28 were obtained from the SWOG Leukaemia Repository at the University of New Mexico, courtesy of Dr Cheryl Willman. Patients #17 and #18 were collected from the Department of Haematology at the Women's and Children's Hospital, North Adelaide, South Australia. All samples were stored in liquid nitrogen until required for analysis. Ethics approval was obtained from the institutions involved in this study.

Table 5.1 Clinical details and karyotypes of the patients studied

F = female M = male The age of the patient is given in years. White cell counts (WCC) are given as $x10^9$/L. FAB = French American British classification. The FAB subtype of AML refers to that outlined in figure 1.2. UM = unclassified myeloid leukaemia due to insufficient material. MX = AML not classified by a central pathologist. M2Eo refers to an M2 AML in which there is abnormal bone marrow eosinophilia. The treatment protocols were as follows: **study 8600: (1)** cytosine arabinoside (AraC) (200mg/m^2/d days 1-7) by continuous infusion (CI) and daunorubicin (DNR) 45mg/m^2 days 1,3,5) **(2)** AraC 2gm/m^2 every 12 hrs x 12 (days 1-6), DNR 45mg/m^2/d days 7-9; **study 9034:** AraC (25mg/m^2/d IV push + 100mg/m2/d CI days 1-7) and idarubicin (12mg/m^2 days 1,2,3); **study 9126:** AraC (3g/m^2/d CI over 3 hrs, days 1-5) and daunomycin (45mg/m^2 CI, days 6-8) plus cyclosporin A as a resistance modifier given prior to or concurrently with daunomycin; **study 9031:** AraC (200mg/M2/d CI days 1-7) and DNR (45mg/m2 days 1,2,3) plus rhG-CSF; **study 8326: (1)** AraC 2gm/m^2 every 12 hrs x 12 (days 1-6), **(2)** AraC 3gm/m^2 every 12 hrs x 12 (days 1-6); **AraC/DNR** AraC (200mg/m^2/d days 1-7) by continuous infusion (CI) and DNR (50mg/m^2 days 1,3,5).

Patient (age/sex)	Karyotype	WCC (%blasts) peripheral blood	FAB Class.	Treatment Protocol (SWOG)
1 (45,F)	46,XX,inv(16)	19.1 (12%)	M1	8600
2 (36,M)	47,XY, +22,inv(16)	190 (44%)	M2	8600
3 (34,F)	46,XX,inv(16)	111.6 (56%)	MX	9034
4 (20,M)	46,XY,inv(16)	27 (46%)	M4Eo	9034
5 (24,M)	47,XY,+21,inv(16)	6.1 (34%)	M2Eo	9034
6 (27,M)	46,XY,inv(16)	21.8 (6%)	M4	9034
7 (24,F)	47,XX,+21,inv(16)	2.7 (34%)	M2Eo	9034
8 (38,F)	46,XX,inv(16)	124 (80%)	M2Eo	9034
9 (25,F)	46,XX,inv(16)	104.5 (28%)	M4Eo	9034
10 (37,M)	46,XY,dup(8)(q24.1;q24.3), inv(16)	49.7 (73%)	M2	9034
11 (31,M)	46,XY,inv(16)	206.4 (68%)	M4	9034
12 (33,F)	47,XX,+8,inv(16)	12.9 (4%)		9034
13 (44,F)	47,XX,+8,inv(16)	28.7 (44%)	M2	9034
14 (45,M)	47,XY,+22,inv(16)	57.1 (37%)	M2	9034
15 (43,F)	46,XX,inv(16)	20.3 (72%)	M4	9126
16 (48,M)	46,XY,inv(16)/ 47,XY,+22,inv(16)	267 (71%)	M2Eo	8600
17 (7,F)	46,XX,inv(16)	26	M4Eo	AraC/Dauno
18 (14,M)	46,XY,inv(16)	60	M4Eo	AraC/Dauno
19 (41,M)	47,XY,+8,inv(16)/ 48,XY,+8,+22,inv(16)	55	UM	9034
20 (51,M)	47,XY,+8,inv(16)	4.7 (7)	M2	9034
21 (34,F)	46,XX,/47,XX,+8,inv(16)	2.7 (42%)	M1	8600
22 (43,M)	47,XY,+22,t(16;16)	15.3 (62%)	M2Eo	9034
23 (23,M)	46,XY,t(16;16)	206.8 (88%)	M4	8600
24 (68,F)	46,XX,t(16;16)	85.3 (46%)	M4	9031
25 (24,M)	46,XY,t(16;16)	36.4 (18%)	M2Eo	9034
26 (65,F)	45,XX,-11,t(16;16)	77.4 (21%)	M2	8326
27 (50,F)	46,XX,del(16q22)	75.7 (26%)	M4	8600
28 (47,M)	46,XY,del(16q12.1q22)	12.6 (34%)	M2	9034
29 (54,F)	46,XX,del(16q22)	27.7 (53%)	M2	8326

5.3.2 Thawing of cryopreserved bone marrow samples

Bone marrow was stored on liquid nitrogen. Before use, samples were removed from the liquid nitrogen and thawed quickly at 37°C and transferred immediately to ten times volume of thaw solution (see 2.2.4). The cells were then left for ten minutes in a laminar flow hood with the cap off to allow for the diffusion of DMSO. The cells were pelleted following centrifugation at 1100 rpm for 20 minutes. The supernatant was removed and the cells washed once in thaw solution and centrifuged at 1100 rpm for 10 minutes. The cells were then resuspended in approximately 1.5mls of thaw solution depending on the size of the cell pellet and a cell count was performed. A viable cell count was also performed using trypan blue stain for the first five samples and the results used as a guide for the remainder of the samples. Where possible, the cells were resuspended at a concentration of $1x10^7$/ml and four slides were made with a glass spreader for each patient sample. Between $4x10^6$ and $1x10^7$ cells were used for the extraction of DNA (as per the method in section 2.3.1.4) and the remainder (approximately 1×10^7 cells) were incubated at 37°C for 60 minutes prior to the extraction of RNA.

5.3.3 Extraction of RNA

All RNA manipulations were performed using specifically prepared disposable centrifuge tubes and filtered Gilson pipette tips. All handling was performed using gloves and where possible dedicated equipment was used. Solutions were prepared using DEPC treated water as per standard methods (Current Protocols in Molecular Biology, 1993). The incubated cells were centrifuged and resuspended in 400µl of sterile PBS in a eppendorf microcentrifuge tube, to which 1ml of TRIzol was added. The mixture was immediately vortexed and 1/10th

volume of chloroform was added. The suspension was then shaken vigorously for 15 seconds and let to stand on ice for 5 minutes. The mixture was centrifuged at 12,000 x g at 4°C for 15 minutes and the aqueous phase drawn off and an equal volume of isopropanol added. This was vortexed and put on ice for 15 minutes to overnight. The precipitate was centrifuged at 12,000 x g at 4°C for 15 minutes. The supernatant was removed and the precipitate washed with 75% ethanol and briefly centrifuged. The precipitate was then dried on ice and resuspended in 11µl of 1mM EDTA. The RNA was then quantitated by spectroscopy and 1µl was run on a 1% agarose gel to check the integrity of the RNA.

5.3.4 Semi-quantitative RT-PCR

5.3.4.1 The reverse transcription reaction

cDNA synthesis was performed using 2µg of the RNA extracted from each patient sample or control sample. 2µg of RNA was added to DEPC treated water to a total volume of 5µl to which was added 2µl of 50ng/µl of random hexamers (GIBCO BRL). This was heated to 90°C for 10 minutes. In a second eppendorf tube were added 0.2µl of 0.1M dithiothreitol, 4µl of five times buffer (supplied by manufacturer), 4µl of 10mM dNTP mix (dATP, dTTP, dCTP, dGTP at neutral pH), and 2.8µl of DEPC treated water. This was warmed to 37°C. The denatured RNA was briefly centrifuged and added to the second eppendorf tube, to which was added 2µl (400U) of Superscript™ reverse transcriptase(Gibco BRL). The reaction was incubated at 37°C for one hour and then the temperature was increased to 95°C for ten minutes to denature the enzyme. 180µl of non-DEPC treated water was then added to the tube which was then stored at 4°C.

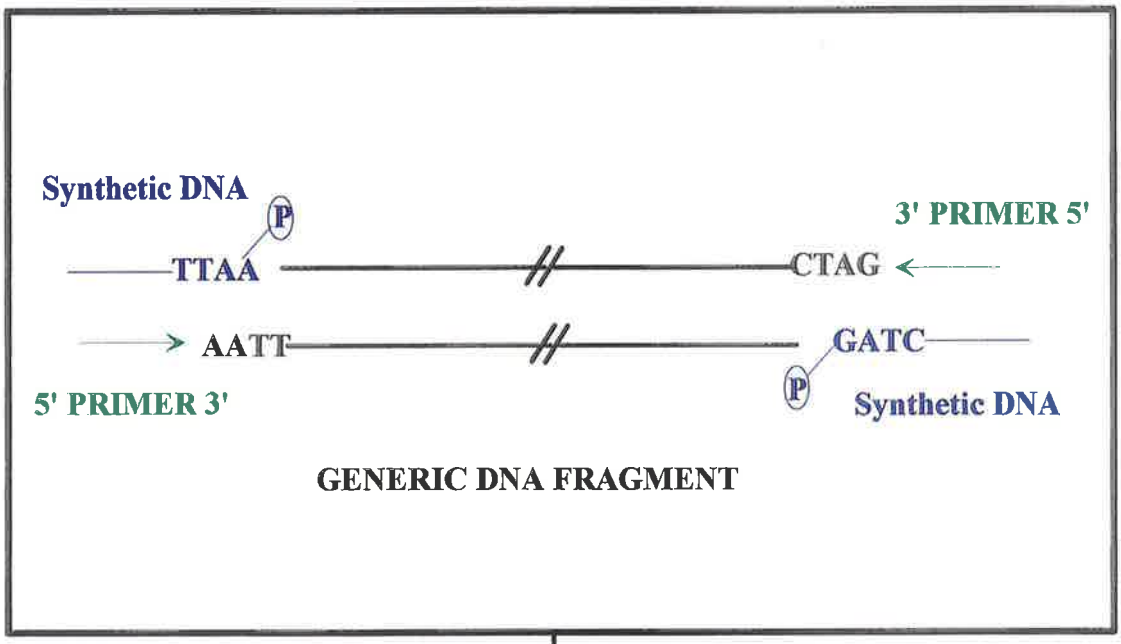
5.3.4.2 Semi-Quantitative PCR

This method was developed with the assistance of Dr. Jim Gerlach and Ms. Leah Lazaruk from the Cancer Research Laboratories, Queen's University, Kingston, Ontario, Canada. RT-PCR is a rapid and sensitive technique for the detection of mRNA expression of a specific gene. The limitations of the use of PCR for a quantitative procedure lie in the many cycles of amplification and the possible kinetic differences between different primer pairs in the amplification of the target sequence. Due to the exponential nature of PCR, small variations in the amplification efficiency can lead to dramatic changes in product yields. The method chosen to address these problems was that of MIMIC™ (Clontech) competitive PCR. This method involved the construction of a non-homologous DNA fragment ligated to the gene specific primers. The non-homologous DNA is constructed so that it is slightly smaller than the target DNA to enable the two products to be simultaneously resolved on an agarose gel. Synthetic DNA fragments complementary to the gene specific primers were constructed on a DNA synthesiser. These synthetic fragments were constructed such that one complementary strand had a 5′ -AATT (EcoRI extension), and the other a 5′- GATC (BamHI extension), both with phosphate groups attached during synthesis- see figure 5.1 The primers for the gene of interest (in this case MRP) were ligated simultaneously to 100ng of the synthetic DNA strands and 100ng of a 0.26kb EcoRI-BamHI v-erbB generic fragment (Clontech) in a 20µl ligation reaction using 20U of T4 DNA ligase (as per the method in section 2.3.2.3.). A 10% portion of the ligation reaction was amplified by PCR using the gene specific primers to generate a PCR MIMIC (Siebert 1993). The amplification step selected out properly ligated competitor DNA as only those ligated to the two synthetic DNA strands will exponentially amplify and generated large quantities of the MIMIC. The amplified MIMIC was then

Figure 5.1 Preparation of PCR MIMIC™

The figure outlines the method for the preparation of a PCR MIMIC™ for competitive PCR. The boxed figure shows the gene specific primers, the synthetic DNA fragments complementary to these primers, and the 0.26kb EcoRI-BamHI v-erbB generic fragment. The synthetic fragments are shown with one complementary strand containing a 5′ -AATT (EcoRI extension), and the other a 5′- GATC (BamHI extension). The primers for the gene of interest were ligated simultaneously to the synthetic DNA and the generic fragment. An encircled P implies an attached 5′-phosphate group. The remainder of the figure outlines the ligation, amplification and purification steps involved in the preparation of the MIMIC™. Experimental details are provided in the text. (Siebert 1993).

Preparation of PCR MIMIC



LIGATION
(2 hours)

Spin Column

PCR
(3 hours)

Spin Column

Competitive
PCR MIMIC

purified by centrifugation and a spin column. The yield of the competitive PCR MIMIC was determined by measuring absorbance at 260nm.

A competitive PCR reaction was prepared using target cDNA and the synthesised MIMIC™ DNA as competitive templates for the PCR. The Target cDNA was synthesised in an initial reverse transcription reaction (as per 5.3.3.2) and added to each PCR reaction in a constant amount. A known quantity of the MIMIC DNA was added to each PCR reaction in a ten fold dilution series, and the resultant PCR products were separated on a 2% agarose gel and visualised with ethidium bromide. The ten fold MIMIC™ dilution series was followed by a two-fold dilution series centred around the estimated quantity of target cDNA. The amount of target sequence present in the synthesised cDNA was then calculated from the results. A schematic diagram demonstrates the expected result in figure 5.2. When the MIMIC DNA is in excess, the MIMIC PCR product predominates. As the concentration of the MIMIC DNA reaches the concentration of the target DNA the two PCR products reach equal quantities and appear as equal intensity bands when visualised following separation on a 2% agarose gel. When the target DNA reaches excess, the target PCR product then predominates as shown in figure 5.2. The reaction conditions were as follows: 1.5µl of 2mM dNTPs, 13.3µl of water, 2.5µl of primer pair mix (containing 150ng of each primer), 2.5µl of 10x buffer (Boehringer), 5µl of cDNA (target) and 0.2µl of Taq polymerase (Boehringer). When MIMIC DNA was added, 1µl of the dilution of the MIMIC was used and 12.3µl of water was substituted for the water volume used above. The cycling conditions were as follows: *95°C(2.5min), 58°C(3min), 72°C(5min) for one cycle; 95°C(45sec), 58°C(1min), 72°C(1min) for 35 cycles; 72°C ten minute extension time.* The oligonucleotide sequences are listed in table 5.2. Only

Table 5.2 Primer sequences for MRP, TFRR and ESD

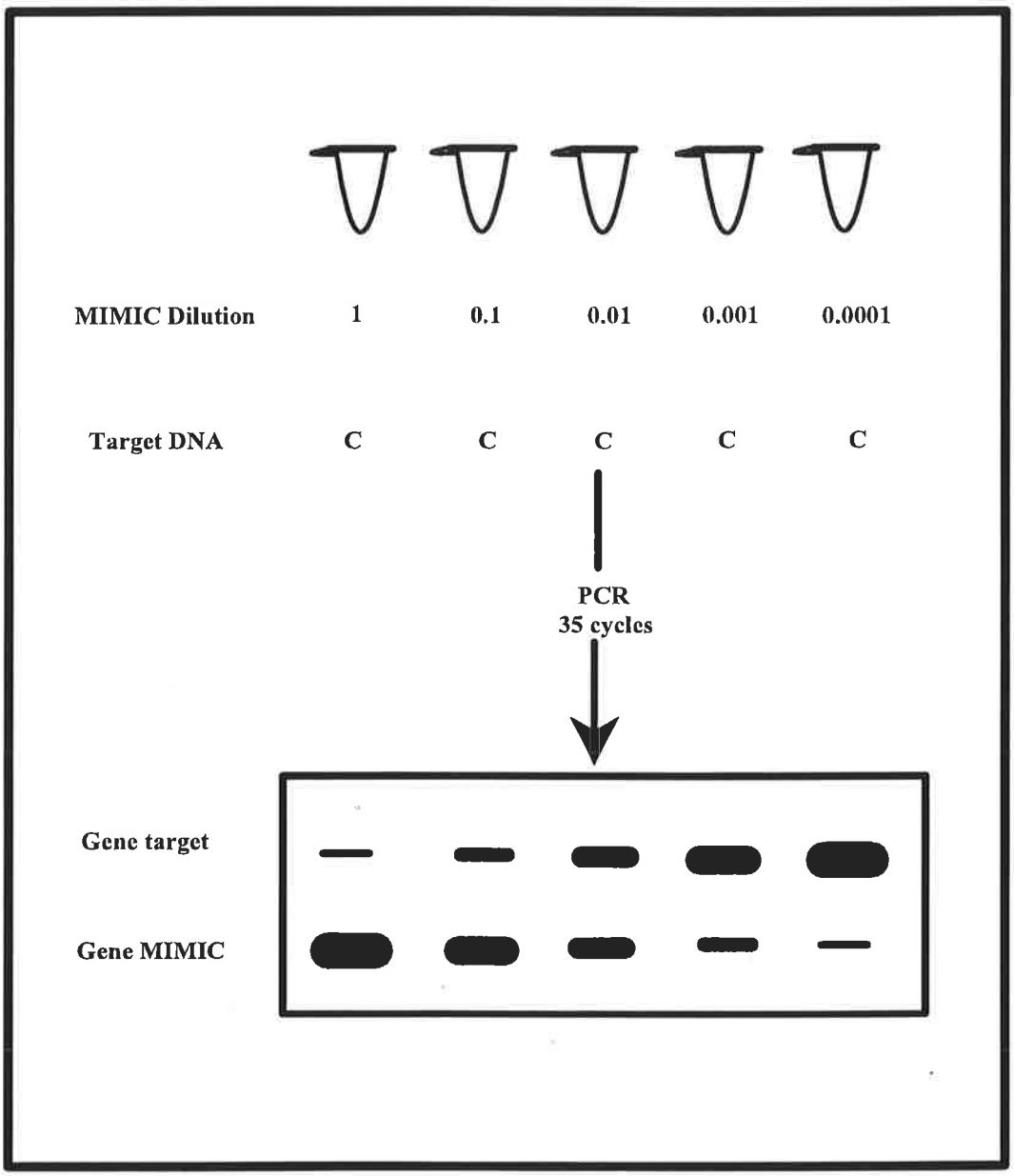
Gene Primer	Sequence
MRP F	AGTGACCCTCTGGTCCTTAAA
MRP R	GAGGTAGAGAGCAAGGATGACTTGC
TFRR F	GGATAAAGCGGTTCTTGGTACCAGC
TFRR R	TGGAGGTAGCACGGAAGAAGTCTCC
ESD F	GGAGCTTCCCCAACTCATAAATGCC
ESD R	GCATGATGTCTGATGTGGTCAGTAA

F denotes forward primer, R denotes reverse primer. Primer sequences for the genes are those used for the competitive PCR following reverse transcription. MRP = multidrug resistance associated protein. TFRR = transferrin receptor. ESD = esterase D.

Figure 5.2 MIMIC™ competitive PCR

Outlined in the figure is the set up procedure and cartoon representation of the products of a competitive PCR, using a synthetic PCR MIMIC™. cDNA is first synthesised from cellular RNA. A ten fold dilution series of the specific gene MIMIC DNA is constructed and a constant amount of sample (**C**) cDNA is added to the PCR reaction. Competitive amplification of gene target cDNA with a known amount gene MIMIC™ DNA added to the PCR reaction, allows quantitation of the level of gene expression in that sample. The intensity of the ethidium bromide stained PCR derived bands, are equal for both the gene target and the gene MIMIC™, when there is an equi-molar amount of gene target and gene MIMIC™ present in the tube, as pictured in the lower section of the figure.

MIMIC PCR


MIMIC Dilution
1
0.1
0.01
0.001
0.0001
Target DNA
C
C
C
C
C
PCR
35 cycles
Gene target
Gene MIMIC

the two fold dilution series was performed in duplicate to reduce the amount of cDNA used for each patient.

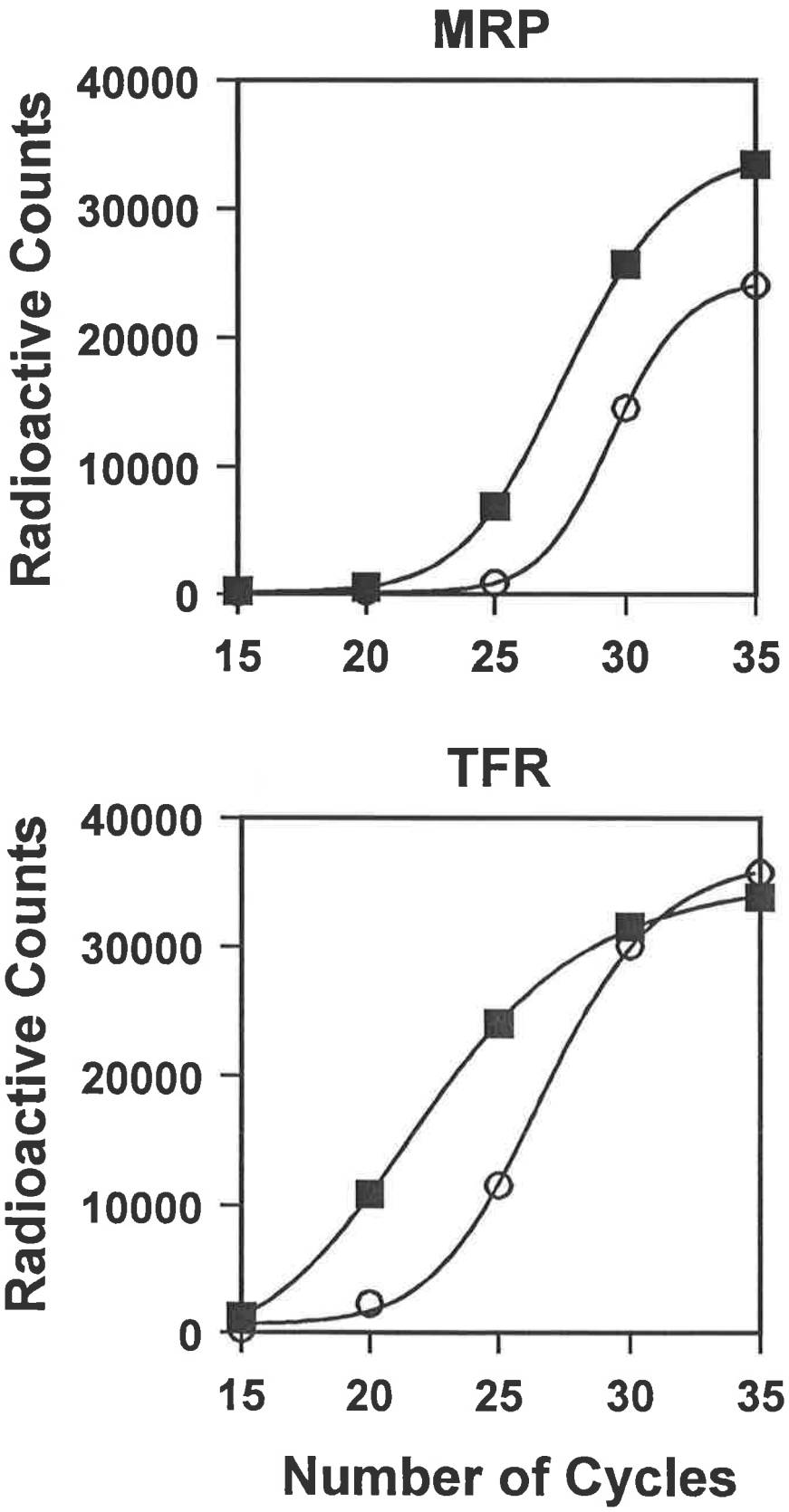
This technique enabled the same primer pair to be used for the amplification of both the cDNA and the control (MIMIC) DNA. This eliminated the problems encountered when more than one primer pair is required to amplify both the target and control cDNA. Different annealing properties of different primer pairs alters the kinetics of the amplification of the target and the MIMIC DNA. However, the kinetics of the templates need also be considered. This is demonstrated in figure 5.3. The data was generated using a similar amount of either target DNA or MIMIC DNA in a series of PCR reactions, removing a tube at five cycle intervals and measuring the amount of product present in each tube. The products were measured by Southern transferring the separated PCR products and hybridising a kinased oligonucleotide (see 2.3.4.1 and 2.3.6.1) of the same sequence as that used in the PCR reaction, to the Southern transferred DNA. The amount of radioactivity for each PCR product was measured using a computer analysed image intensifier. The slope of the two curves is equivalent at each 5 cycle interval demonstrating that the kinetics of the amplification of the two templates is comparable. The absolute value of the counts for each 5 cycle increment during the exponential phase of the PCR reaction, is dependent on the amount of template DNA present at the beginning of the reaction.

The housekeeping genes chosen as controls for the quality of the cDNA synthesis were Esterase D (ESD) and Transferrin Receptor (TFRR). Both controls were initially analysed in establishing the technique however, it was found that the results for ESD and TFRR were

Figure 5.3 Kinetic study of the PCR amplification of gene target and gene MIMIC™

The PCR amplification using the gene target and the gene MIMIC™ for *MRP* and *TFRR* is shown in graphic form. Data is derived from PCR reactions stopped at 5 cycle intervals beginning at 10 cycles (horizontal axis). The products of the PCR reactions were separated on a 2% agarose gel and transferred to a nylon membrane by Southern blotting. The membrane was probed with a $\gamma^{32}P$ labelled oligonucleotide used in the PCR reaction. The radioactive signal (counts) was quantitated for each PCR product using an image amplifier system (Packard, USA) and has been plotted on the vertical axis. The gene target is represented by the dashed line and the gene MIMIC™ is represented by the solid line.

KINETIC STUDIES



similar within the population of control and patient samples. The annealing temperatures of the MRP and TFRR primers were compatible, allowing the experiments to be performed simultaneously in the one thermocycler. Therefore for the sake of conserving cDNA and for convenience, TFRR was chosen as the housekeeping gene to be used in the semi-quantitative analysis of MRP expression.

5.3.4.3 Southern transfer and hybridisation, and quantitation using computer analysed image intensification

The PCR reactions were separated on a 2% agarose gel and visualised with ethidium bromide. The PCR products were then Southern transferred as in 2.3.5. The membranes were then hybridised at 42°C to the forward primer of the PCR reaction labelled with γ^{32}P by a T4 polynucleotide kinase reaction (2.3.4.1.). This method was chosen to ensure that the same quantity of γ^{32}P-dATP was incorporated into each strand of DNA synthesised. Incorporation of α^{32}P-dCTP in the PCR reaction may result in different activities dependent on the amount of α^{32}P-dCTP incorporated in each strand rather than the number of synthesised DNA strands present. The results were quantitated using a computer analysed image intensifier and the ratio of counts for each target/MIMIC product was calculated. This was compared to the visual image of the ethidium bromide stained gel and the autoradiograph for each reaction series. The results of the ratios of target/MIMIC were then graphed using the FPWIN statistical package, FigP Software Corporation, Durham, NC, USA and the cDNA value for a ratio equalling unity, was calculated. This value is obtained when equi-molar amounts of both template DNAs are present in the PCR reaction. An example of the

competitive PCR amplification of *MRP* and the graphic analysis of the data is shown in figure 5.4a & b.

In addition to the measurement of the expression of *MRP*, the expression of a control housekeeping gene (Transferrin Receptor: *TFRR*) was quantitated using the same technique as described for the MRP MIMIC with a specifically constructed *TFRR* MIMIC. The amount of *MRP* and *TFRR* cDNA present in each cDNA synthesis reaction was calculated and the final result was expressed as a ratio: *MRP/TFRR*. The use of the housekeeping gene controlled for variation in the efficiency of the cDNA synthesis. This ratio was calculated for patient samples, normal bone marrow samples, normal flow sorted CD34+ cells, monocytes and peripheral blood lymphocytes. The cell line U937, and three drug resistant *MDR1* expressing leukaemic samples were also examined. The flow sorting of the CD34+ cells was performed by Dr. David Haylock of the Hanson Centre for Cancer Research, Leukaemia Research Unit. The neutrophils were purified and the monocytes elutriated by Dr Jeffrey Barbara of the Hanson Centre for Cancer Research, Dept. of Immunological Research, Adelaide.

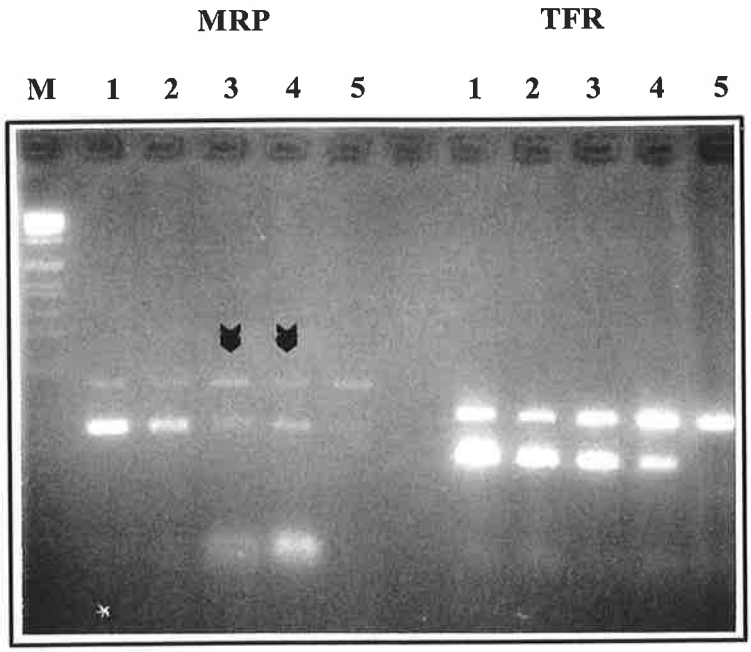
5.3.5 Interphase cytogenetic analysis of inv(16) leukaemic cells

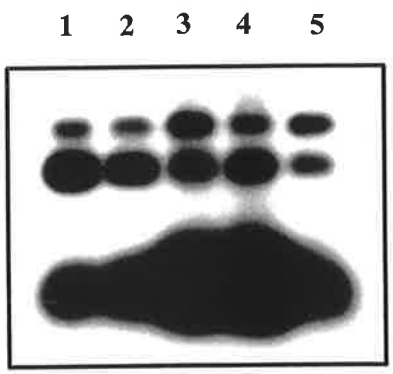
The slides spread from the thawed patient bone marrow samples were immediately fixed in 3:1 methanol:acetic acid by standing in a filled Coplin jar overnight. The following morning the slides were air dried and stored in a sealed container at -20°C until required for use. Three to four slides were hybridised per sample. Five normal bone marrow samples from patient and volunteer sources were used to obtain a normal range for the hybridisation of the

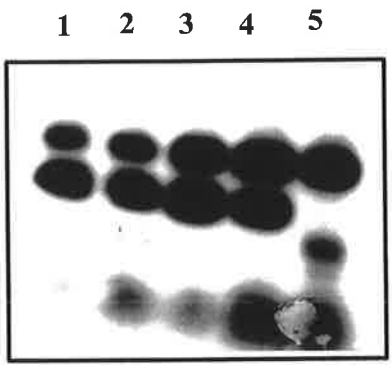
Figure 5.4a Competitive PCR amplification of *MRP* and *TFRR* cDNA for patient #18

MRP and *TFRR* gene expression for patient #18 is represented in the top figure. PCR amplification products were separated on a 2% agarose gel and stained with ethidium bromide. The upper bands represent the gene products and the lower bands represent the MIMIC™ products. The lanes contain the following: MRP MIMIC™ - lane 1: 10^{-6}, lane 2: 10^{-7}, lane 3: 2.5 x 10^{-8}, lane 4: 5 x 10^{-8}, lane 5: 10^{-8}; TFRR MIMIC™ - lane 1: 10^{-6}, lane 2: 10^{-7}, lane 3: 5 x 10^{-8}, lane 4: 2.5 x 10^{-8}, lane 5: 10^{-8}. The arrowed lanes are to mark that the samples of these two lanes were loaded in reverse order of quantity of MIMIC™ added. This is visually evidenced by the lack of progressive reduction in intensity of the lower bands. The lower two figures represent autoradiographs of the same gel (for experimental details see 5.3.4.3). The left figure representing the amplification of *MRP* and the right figure that of *TFRR*. Results were quantitated on an image amplifier (Packard).

Competitive PCR





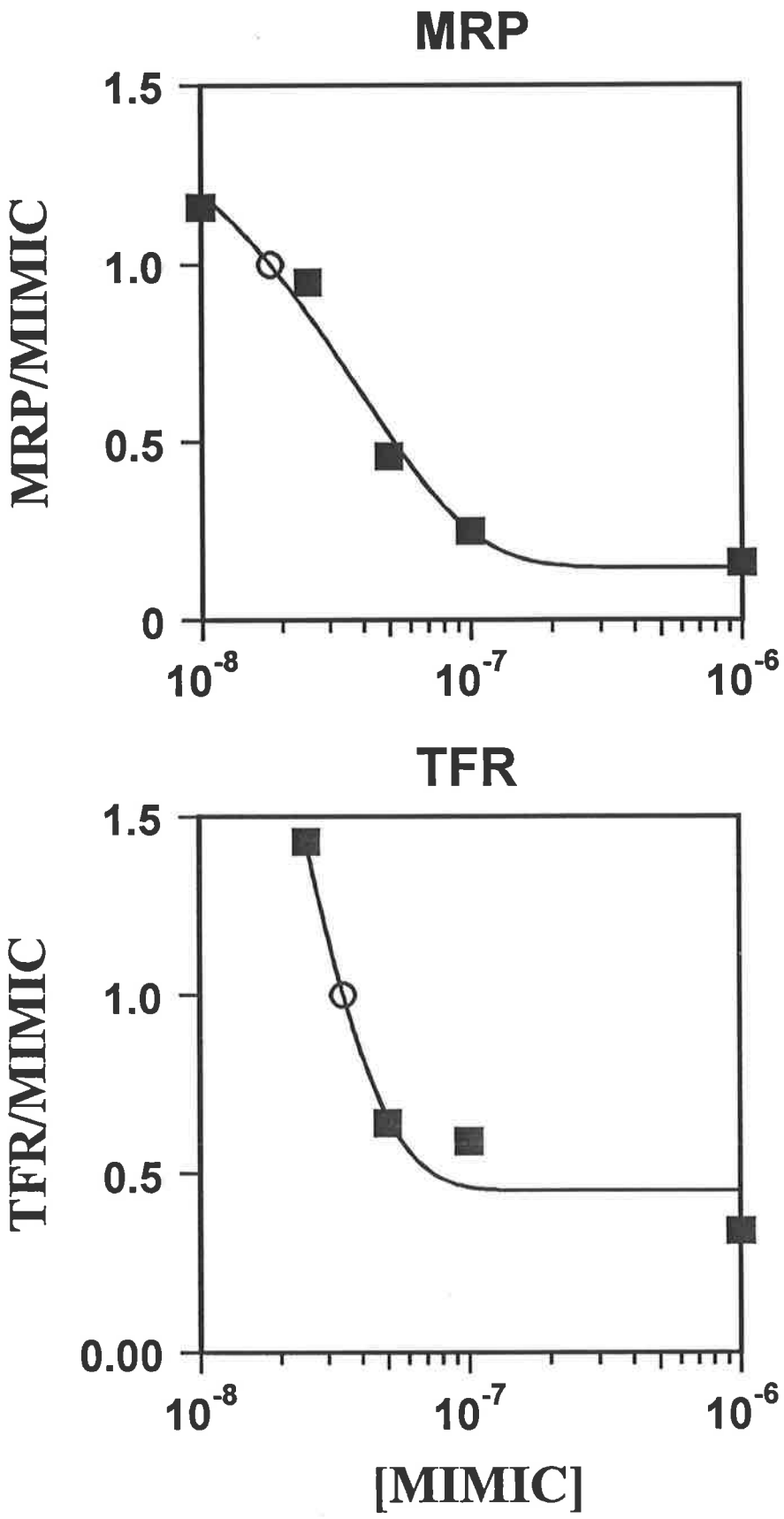


Autoradiographs of MRP and TFRR

Figure 5.4b Graphic data of the competitive PCR for patient #18

[MIMIC] = MIMIC™ concentration. MRP = multidrug resistance associated protein. TFR = transferrin receptor. MIMIC concentrations are plotted on a logarithmic scale. MRP/MIMIC = the ratio of the radioactive counts measured for each MRP gene/MIMIC™ PCR reaction. TFR/MIMIC = the ratio of the radioactive counts measured for each TFR gene/MIMIC™ PCR reaction. The radioactive count for the gene target product is compared with that for the gene MIMIC™ product in the same reaction and a ratio is calculated. These are shown as a linear scale on the vertical axis. A curve of best fit has been calculated. A gene/MIMIC™ ratio of one is reached when there are equimolar amounts of MIMIC™ and gene target DNA present as templates, at the start of the thermocycle reaction.

EXPRESSION


MRP
MRP/MIMIC
1.5
1.0
0.5
0
10^{-8}
10^{-7}
10^{-6}
TFR
TFR/MIMIC
1.5
1.0
0.5
0.00
10^{-8}
10^{-7}
10^{-6}
[MIMIC]

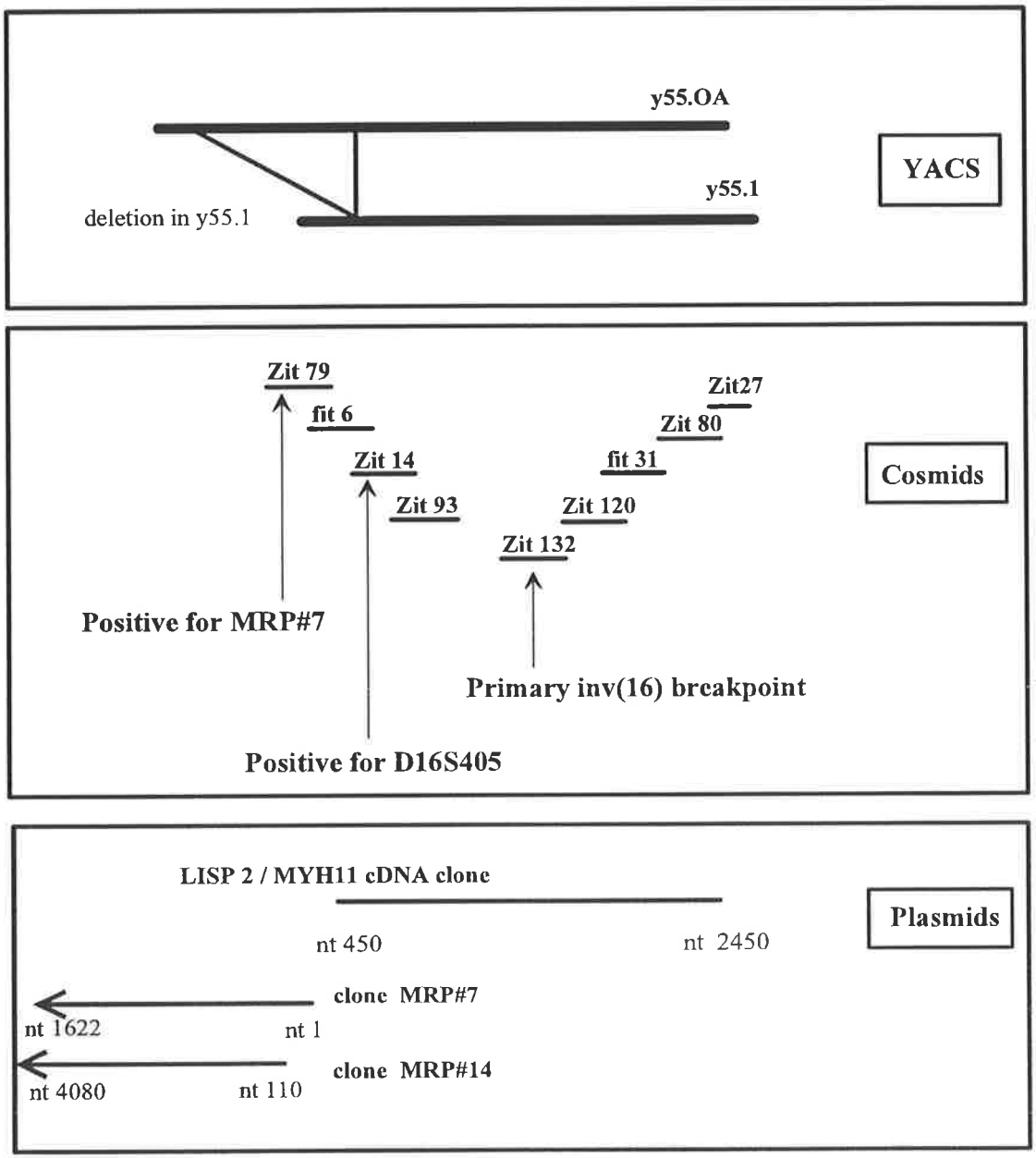
MRP probe to interphase nuclei. Several *MRP* genomic and cDNA probes were tested in FISH experiments to metaphase spreads and interphase nuclei before MRP#14 was finally selected as the probe of choice. This probe demonstrated low background using FISH techniques and hybridised specifically to 16p13.13. (see figure 4.6). The *MRP*#14 probe was nick translated with biotin-14-dATP and hybridised *in situ* at a final concentration of 20ng/μl to interphase cells from patients with the chromosome 16 rearrangements. The nuclei were counter stained with propidium iodide. For the analysis of each sample 2-3 slides were examined independently by two experienced microscopists (the student and Mrs. Helen Eyre in the Dept. of Cytogenetics and Molecular Genetics at the W&C H). One hundred nuclei were scored for zero, one, two, three, four or >four fluorescent signals. The scores were then tallied, compared with control samples and the log of the individual scores were analysed using a Student's t test.

5.3.6 Southern analysis of restricted leukaemic DNA

DNA extracted from leukaemic cells was digested with the restriction endonucleases EcoRI and KpnI using standard conditions (2.3.3.1). Spermidine was not used in the KpnI reactions as this caused precipitation of the DNA. The restricted DNAs were then Southern transferred and hybridised to labelled DNA probes positive for the region within 10kb proximal to the short arm breakpoint region of the inv(16) leukaemias (see methods in sections 2.3.4.2 & 2.3.5). These probes were the 2kb *MYH11* cDNA probe LISP2, and a specifically prepared 1.2kb AccI restriction fragment from the zit132 cosmid (figure 5.5). The probes LISP2 and zit132 were supplied by Prof. Martijn Breuning and Dr. Bert van der Reijden, of Leiden University, the Netherlands.

Figure 5.5 Genomic and cDNA clones of *MYH11* and *MRP*

Schematic diagram showing the relationships of the YAC and cosmid contigs containing the *MYH11* gene and the short arm inv(16) breakpoint region. Approximate distance separating cosmids zit132 and zit79: 150kb, represents the distance from the primary breakpoint region of *MYH11* to the 5' end of the *MRP* gene. zit79 is the most centromeric cosmid. cDNA clones for *MYH11* (LISP2) and *MRP* (MRP#7 and MRP#14) are shown. zit 79 is positive for a unique 218 bp fragment of MRP#7. Zit 14 is positive for the AC polymorphic repeat, *D16S405*. This figure is the same as figure 4.2 but is included again here for the convenience of the reader.


y55.OA
y55.1
deletion in y55.1
YACS
Zit 79
fit 6
Zit 14
Zit 93
Zit 132
Zit 120
fit 31
Zit 80
Zit27
Cosmids
Positive for MRP#7
Positive for D16S405
Primary inv(16) breakpoint
LISP 2 / MYH11 cDNA clone
nt 450
nt 2450
Plasmids
clone MRP#7
nt 1622
nt 1
clone MRP#14
nt 4080
nt 110

5.3.7 RT-PCR for the detection of hybrid *CBF-MYH11* transcripts

The hybrid transcripts representing the fusion product between 5′ CBFB/MYH11 3′, are detectable in all cases of inv(16) by RT-PCR. Results of the transcripts found in patients #1 - #7, #9, #16, #22 #23, #25 were provided by Dr Cheryl Willman from whom the cryopreserved bone marrow samples were obtained. The methods used were those outlined by Shurtleff (1995). The remaining fusion transcript results were not available at the time of thesis preparation.

5.3.8 Statistical analysis of data

Survival distributions were estimated by the method of Kaplan and Meier (1958). Since the number of patients was limited for comparing those with and without deletion of the *MRP* gene and those in which the *MRP/TFRR* expression ratio was >3 vs <3, exact p-values were calculated from the permutation distributions of Wilcoxon rank sum and log rank statistics as appropriate, using the commercially available StatXact Turbo program (Cytel Software Corporation, Cambridge, Massachusetts, USA). Applying a Rank Sum analysis to the data reduced the variance of the sample overall and was an appropriate test to use for the analysis of this data. The two-sample or Student's t-test was calculated on the log values of the *MRP/TFRR* expression ratios, to normalise the data.

5.4 Results

5.4.1 Clinical details of patients

Twenty nine patients with AML associated with clonal rearrangements of chromosome 16 were retrospectively studied. This included twenty one patients with inv(16)(p13q22.1); five patients with homologous rearrangements of chromosomes 16: t(16;16)(p13;q22.1) and three patients with del(16)(q22.1). Their FAB subclass diagnoses and other clinical data are included in table 5.1. A slide from each of the stored bone marrows was prepared and stained with May Grunweld Geimsa stain, and the percentage of blasts present in each frozen bone marrow sample calculated. This blast percentage often differed from the bone marrow blast count as the cryopreserved bone marrow had first been subjected to a ficoll density gradient to remove red cells and mature granulocytes. In patients #11, #15, #20, #22 and #29 high numbers of eosinophils remained in the stored bone marrow due to their abnormal density characteristics, however not all of these samples were classified as having abnormal bone marrow eosinophilia. This data is shown in table 5.3 along with an abbreviated immunophenotype analysis obtained from Dr Cheryl Willman of the University of New Mexico Cancer Center. The immunophenotype analysis revealed that the majority of samples were CD34+, HLA-DR+, CD38+, CD44+, CD33+, CD11a indicative of a persisting primitive phenotype with some differentiation toward a concurrently expressed myeloid/ monocytoid phenotype. Interestingly, CD2 was not expressed in this cohort of patients as compared with the report of Adriaanson (1993).

Table 5.3 Immunophenotype analysis and additional patient information

BM = bone marrow. CD = cluster of differentiation antigen. DR = HLA-DR = human leucocyte antigen-D related which is present on primitive myeloid cells. CD34 denotes a pluripotent stem cell/immature haematopoietic cell. CD33 denotes myeloid differentiation. CD38 denotes early monocyte differentiation (in the setting of M4Eo), but is also present on activated T cells. CD11a denotes a β_2 integrin present on neutrophils, monocytes and macrophages. CD44 is present on monocytes. AML = acute myeloid leukaemia. MPD = myeloproliferative disease. Where the word "eosinophils" appears in the fourth column, this implies abnormal eosinophils were seen in the cryopreserved bone marrow sample.

Patient	BM Blast % (blast % in frozen sample)	Immunophenotype	Comments
1	70 (70%)	DR,CD34,CD13,	
2	76 (80%)	DR,CD34,CD11a	25% viable
3	90 (95%)	DR,CD34,CD38,CD13,CD11a,CD44	
4	71 (80%)	CD34,CD13,	25% viable
5	37 (70%)	DR,CD34,	
6	58 (80%)	DR,CD38, CD18,CD44,CD11c	
7	31 (90%)	DR,CD13 (CD34)	50% viable
8	81 (90%)	DR,CD34,CD38,CD33,CD11a,CD44	very hypocellular BM, 50%viable
9	40 (80%)	DR,CD34,CD11a,	40% viable
10	83 (90%)	DR,CD34, CD38,CD13,CD11a,CD44	
11	33 (95%)	DR,CD34, CD38, CD33,CD11a	Eosinophils, 30%viable
12	41(80%)	DR,CD34,CD38,CD33,CD44	20% viable
13	88 (90%)	DR,CD34,CD38,CD13,CD11a,CD44	50%viable
14	70 (70%)	DR,CD34,CD38,CD33,CD11a CD44	50% viable
15	81 (80%)	DR,CD34,CD38,CD33,CD11a CD44	Eosinophils, relapsed AML, 10% viable
16	66 (70%)	DR,CD34,CD13,	
17		DR,CD34,CD38,CD33,CD11a CD44	
18		DR,CD34,CD38,CD33,CD11a CD44	
19	78	not available	
20	52	DR,CD34,CD38,CD33,CD11a CD44	Eosinophils
21	90 (90%)	DR,CD34,CD33	
22	70 (90%)	CD13	Eosinophils
23	75 (95%)	DR,CD34,CD13,CD11a	40% viable
24	20 (65%)	DR,CD38,CD13,CD11a,CD44	10-15% viable
25	43 (80%)	DR,CD34,CD11a	25% viable
26	24 (80%)	DR,CD34	AML secondary to MPD, 25% viable
27	70 (75%)	DR, CD34, CD13,CD11a,	
28	18 (60%)	DR,CD34,CD38,CD33,CD11a,CD44	Very hypocellular BM
29	67 (90%)	DR,CD34, CD11a	Eosinophils, secondary AML

Unfortunately, the clinical outcome for over half of the patients included in the study could not be obtained from the SWOG data base due to the data not being released until July 1997. This made it impossible to correlate the results obtained with the duration of first remission or time to failure (which included failed induction therapy) as was possible for the cohort used in chapter 4 of this thesis. Where available clinical outcome is documented in table 5.5.

5.4.2 RT-PCR

5.4.2.1 Patient sample quality

RNA was extracted from thawed bone marrow samples from all patients as listed in 5.1. The quality and the quantity of the RNA extracted was sub-optimal in a number of cases (patient numbers #10, #11, #13, #15, #19, #20, #23-#26). This appeared to be due to a large amount of cell death in the SWOG samples received from New Mexico. The cell counts for all samples revealed an average cell death of approximately 30% compared with the patient samples listed above, where the cell death varied from 60% to 75%. This resulted in suboptimal quantities and quality of RNA and DNA extracted from the patient samples, compared with the control cells used from other sources.

5.4.2.2 Semi-quantitative PCR

cDNA was synthesised from RNA samples extracted from all patients listed in table 5.1 and from control cells listed in table 5.4. RT-PCR was successfully performed on those patients as listed in table 5.5. The *MRP/TFRR* expression ratios for each patient are shown. Figure 5.4 shows an example of the RT-PCR products of patient #18 as seen on an ethidium bromide stained 2% agarose gel and the autoradiograph of that gel probed with end labelled

Table 5.4 Analysis of the expression of the MRP gene in selected haemopoietic cell types

Control Samples	Control Cell type	MRP/TFRR Ratio (normalized)
1	CD34+ cells (1a) CD34+ cells (1b)	1 1
2	Bone Marrow Mono-nuclear cells 1	<1
3	Bone Marrow Mono-nuclear cells 2	<1
4	Peripheral Blood MNC 1	2
5	Peripheral Blood MNC 2	2
6	Peripheral Blood MNC 3	4
7	Monocytes	0.5
8	Peripheral Blood Stem Cell Harvest	1
9	Drug Resistant Leukaemic Cells (P-glycoprotein+) 1	1
10	Drug Resistant Leukaemic Cells (P-glycoprotein+) 2	1
11	Drug Resistant Leukaemic Cells (P-glycoprotein+) 3	1
12	U937 cells	0.5

P-glycoprotein+ denotes acute myeloid leukaemic cells which express P-glycoprotein. U937 cells are a monoblastoid leukaemic cell line.

Table 5.5 *MRP* deletion status, *MRP* expression and clinical outcome

MRP/TFRR denotes the ratio of the quantitated values for *MRP* gene expression and *TFR* gene expression. inv(16) and t(16;16) denote the inversion or translocation of chromosome 16 associated with AML. The number of hybridisation signals seen in interphase nuclei from the leukaemic patients and the control bone marrow samples is expressed as a 1:2 signal ratio average, being the average score of at least three slides for each patient. The probe used was MRP#14. "Metaphase" implies metaphase chromosome analysis was performed using the same probe (MRP#14) as for the interphase hybridisations (4.4.3). "Southern" implies gene dosage was confirmed by Southern analysis as per 4.4.6. The deletion status of the three del(16q) patients is not strictly known, however, no chromosome 16 short arm abnormalities were detected therefore it is likely that they possess two intact *MRP* genes.

Patient	MRP/TFRR ratio	Karyotype	MRP deletion status (1:2 signal ratio ave.)	Clinical Outcome (days)	
1	5	inv(16)	not deleted (1.60)	Deceased	405
2	0.2	inv(16)	deleted (3.16)	Deceased	492
3	2	inv(16)	not deleted (0.75)	Alive	963
4	2	inv(16)	not deleted (1.60)	Alive	807
5	1	inv(16)	not known	Alive	873
6	0.4	inv(16)	not known	Alive	906
7	0.5	inv(16)	not known	Alive	128
8	0.5	inv(16)	deleted (3.80)	Deceased	153
9	1	inv(16)	not known	Deceased	721
10	-	inv(16)	not deleted (1.00)	Alive	589
11	-	inv(16)	not deleted (0.77)	Alive	118
12	0.02	inv(16)	deleted (4.67)	Alive	28
13	-	inv(16)	not deleted (0.325)	Alive	632
14	5	inv(16)	not deleted (1.61)	Deceased	150
15	-	inv(16)	not deleted (2.14)	Alive	664
16	1	inv(16)	not deleted (metaphase)	Deceased	11
17	0.4	inv(16)	not deleted (metaphase)	Alive	282
18	0.1	inv(16)	deleted (metaphase)	Alive	1745
19	-	inv(16)	not deleted (0.56)*	Deceased	375
20	-	inv(16)	not deleted (0.55)	-	-
21	2	inv(16)	not deleted (Southern)	Deceased	486
22	2.5	t(16;16)	not deleted (0.405)	Alive	1134
23	10	t(16;16)	not deleted (0.675)	Deceased	8
24	-	t(16;16)	not deleted (0.59)	Deceased	359
25	0.5	t(16;16)	not deleted (0.998)	Alive	1003
26	-	t(16;16)	not deleted (0.67)	Deceased	28
27	10	del(16q22)	not known	Deceased	221
28	-	del(16q12.1q22)	not known	Alive	336
29	4	del(16q22)	not known	Deceased	254

Controls	MRP/TFRR ratio	Karyotype	MRP deletion status
1	<1	46,XX	not deleted (1.07)
2	<1	46,XY	not deleted (0.64)
3	<1	46,XX	not deleted (1.33)
4	-	46,XY	not deleted (0.78)
5	-	46,XX	not deleted (0.67)

* Patient DNA was also analysed by Southern demonstrating no MRP deletion had occurred.

oligonucleotides. The result of visual estimation from the ethidium bromide gel of the equivalence point, representing equi-molar concentrations of the MIMIC and the target DNA present at the start of the PCR reaction, was identical to the result seen on autoradiograph in all cases except one (pt. #4) where there appeared to be sub-optimal hybridisation of probe to the nylon membrane possibly due to poor transfer of the DNA. The PCR result was checked and the hybridisation was repeated however insufficient cDNA prevented repeating the entire experiment for that patient.

The radiographic signal from the hybridised membrane was quantitated using image quantitated computer assistance in which signal was counted within a standardised area of the screen for each PCR product. A ratio was generated for each target/mimic pair and plotted on a graph. The equivalence point (relative to the appropriate MIMIC DNA) was calculated for *TFRR* and *MRP* for each patient from the curve of best fit for each set of data and the results expressed as the ratio of *MRP/TFRR* (table 5.5). The expression of *MRP* relative to *TFRR* for the 99% pure CD34+ cells was 10 and this was taken as the standard for the entire study, nominating this value as a ratio of 1, to which all other results were compared.

Of the 20 patients in whom results of the relative expression of *MRP* to *TFRR* could be generated, eight patients had a ratio of greater than 1, four patients had a ratio equalling 1, and 8 patients had ratios less than 1. Of the seven patients in whom the ratio was greater than 1, two were t(16;16) and two were del(16q) and this represented all of the patients with del(16q) abnormalities tested. The highest ratios detected were those of patients #22 (t(16;16)) and #26 (del(16q)), each with ratios of 10. This implied a 10 fold increase in *MRP*

expression in these leukaemic cells over normal CD34+ cells. In all but one case where the *MRP/TFRR* ratio was less than 1 the associated chromosomal abnormality was inv(16), the outstanding case contained a t(16;16) chromosomal abnormality.

5.4.3 Assessment of the Deletion Status of the inv(16) Cohort

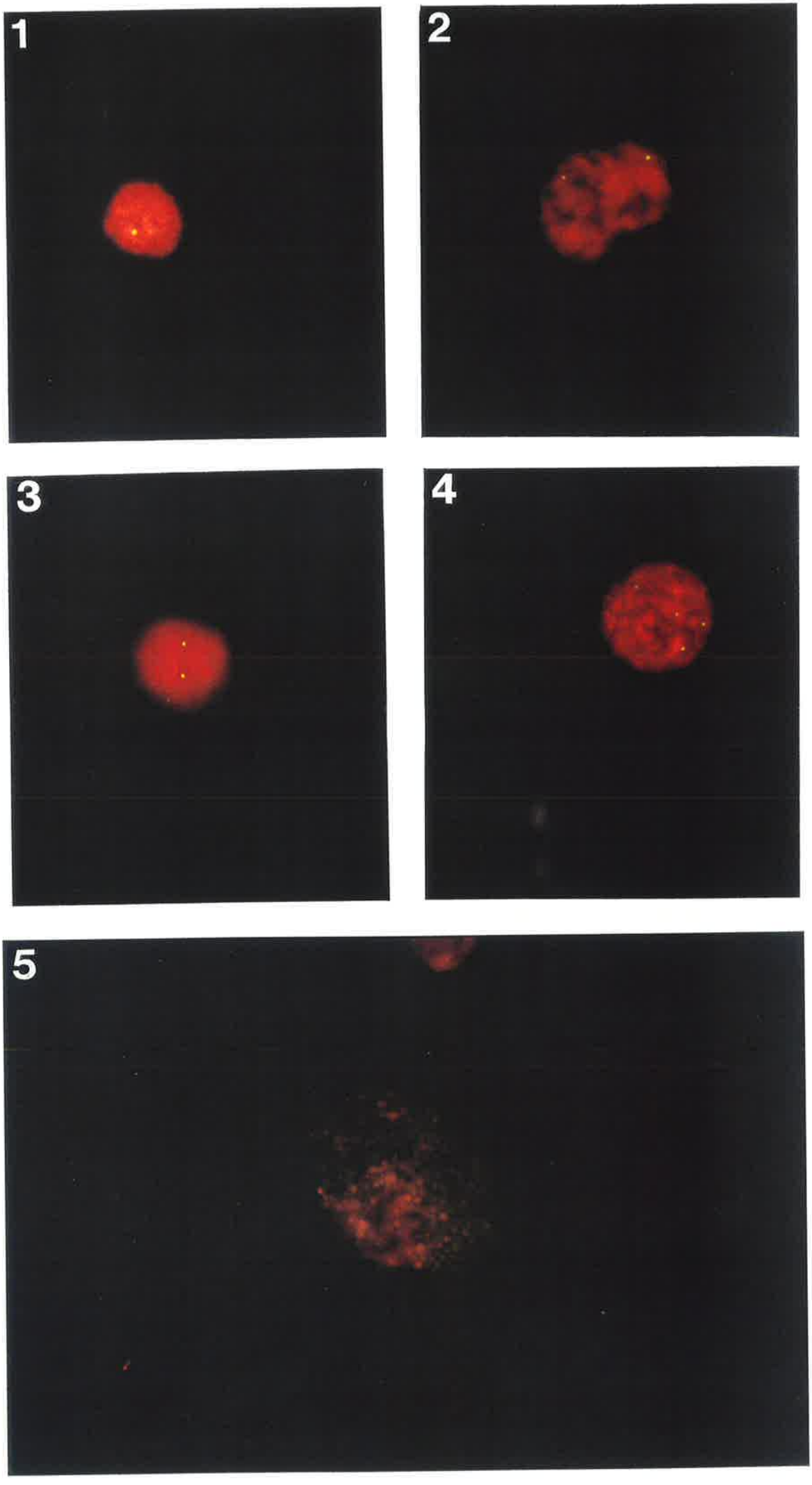
5.4.3.1 Interphase FISH Analysis

Interphase nuclei from 24 of the 29 patients were examined by FISH using the cDNA probe MRP#14. Patients# 5,#6,#7 and #9 were unable to be examined due to insufficient cell numbers. The signal obtained with the probe MRP#14 showed little background hybridisation (figure 5.6). The number of cells per 100 cells counted, which hybridised to the probe producing a signal (percentage hybridisation) varied from 5% to 63%. Where the hybridisation was less than 20% the results were not included in the analysis as this level of hybridisation was considered to be too low to provide accurate information. In these cases the experiment was repeated. Poor hybridisation appeared to be a problem with individual slides rather than individual patient samples and in all cases, three slides with adequate numbers of nuclei with fluorescent signal could be examined. Three normal bone marrow controls were analysed as well as two remission samples from the patient material.

For each sample analysed by interphase FISH, the number of hybridisation signals was scored as zero, one, two, three and four or more and the results are presented in table 5.5. In the control samples there were either approximately equal numbers of nuclei with 1 or 2 hybridisation signals or 2 hybridisation signals per nucleus predominated. The ratio of the number of nuclei scored with 1 compared with 2 hybridisation signals varied from 1.33 - 0.3

Figure 5.6 Interphase FISH analysis using the probe MRP#14

Interphase nuclei are shown, hybridised to FITC labelled MRP#14. Photograph #1 shows a single hybridisation signal, #2 - two signals, #3 - three signals and #4 - four signals. The lower photograph is that of an abnormal eosinophil, showing auto-fluorescence of granules. The fixation and wash process removed the cytoplasm of all other cell types. Greater than two fluorescent signals may occur when cells are fixed during the nuclear DNA replication phase prior to cell division.


1
2
3
4
5

with a mean of 0.98+/-0.132 (standard error of the mean-SEM) with a 95% confidence interval (CI) of 0.53-1.26 in the control samples and the remission specimens. Four patients (patient numbers #2, #8, #12 and #18) were considered to carry deletions of *MRP* based on the results as having 1:2 hybridisation signal ratios of greater than three, implying greater than three fold excess of one hybridisation signal over two signals. This was outside the range found for 1:2 hybridisation ratios for the control samples. Also, the interphase FISH analysis for patient #19 could be compared with Southern analysis of DNA from that patient and the two results were concordant. In each case scored as a deletion of one *MRP* allele, the chromosome 16 abnormality was the inv(16). No t(16;16) chromosomal abnormalities examined were found to carry a deletion of *MRP*. This implied that 19% of the inv(16) cohort carried a deletion of one *MRP* allele and in each case, the *MRP/TFRR* expression ratio was less than one (0.2, 0.5, 0.02 and 0.1 respectively) implying lower expression of *MRP* when compared with normal CD34 cells. In the remaining inv(16) and t(16;16) cases where the deletion status could be determined, corresponding *MRP/TFRR* expression ratios were 2 (pt. #3), 5 (pt. #14), 0.5 (pt. #17), 2 (pt. #21), 2.5 (pt. #22), 10 (pt. #23), and 0.5 (pt. #25).

5.4.3.2 Southern Analysis

Patient DNA restricted with EcoRI or KpnI was hybridised to two probes containing DNA centromeric to the *MYH11* short arm breakpoint of the inv(16). The first probe used was a 1.2 kb AccI restriction fragment of the genomic cosmid zit 132 (refer figure 5.5). This cosmid contains the breakpoint region of the *MYH11* gene and includes sequences both proximal and distal to the breakpoint. The AccI fragment was equivalent to a probe used by Marlton et al (1995), pNE5A, to demonstrate deletions proximal to the short arm breakpoint

of the inv(16). This probe was situated within 10kb of the primary breakpoint region of the *MYH11* gene. The second probe was a 2kb cDNA probe of the *MYH11* gene, LISP2, which has been used by Poirel (1995) to demonstrate the existence of a deletion of the short arm of chromosome 16 associated with the inv(16) (refer also to figure 5.5). For both probes, the expected findings were a normal germline fragment of 14kb in the EcoRI digest together with a rearranged band in inv(16) patients, usually of increased size (possibly greater than 25kb). In the inv(16)/deletion cases this rearranged band would be absent. In both the EcoRI and the KpnI digested patient DNA, the probes failed to yield any bands of the proposed size in any of the inv(16) cases. Both probes had been previously used with success on cosmid DNA and normal total human genomic DNA restriction digested with these enzymes. However despite trying a number of different hybridisation and wash conditions no specific bands were detected, only increased lane background. A probable explanation for this was that significant DNA degradation of the patient samples had occurred and related to the degree of cell death seen in the cryopreserved samples. This would be more evident in the larger sized DNA fragments required for the hybridisation of these probes than in DNA fragments less than 10kb. No data was able to be obtained which could further corroborate the interphase *in situ* results obtained.

5.4.4 RT-PCR for the Detection of Hybrid Transcripts

The results provided by Dr Cheryl Willman are outlined in table 5.5. The majority of the transcripts were of type A (as shown in figure 3.17) except for patient #7 whose transcript type was of type E, patient #5 - type F and patient #21 - type H.

5.4.5 Statistical Analysis of Results

The interphase FISH data was analysed such that patients #2, #8, #12, and #18 were classified as Group I as carrying a deletion of one *MRP* gene while patients #1, #3, #4, #10, #11, #13-#15, #19, #20, #22-#26 were classified as Group II, having two copies of the *MRP* gene. The figures were analysed using a Student's t test with unequal variance. Within the control group the mean of the 1:2 signal ratio was 0.98+/-0.132 (standard error of the mean -SEM) with a 95% confidence interval (CI) of 0.53-1.26. Group I had a mean of 3.87 +/-0.437 (SEM) with a 95% CI of 1.99-5.76. Group II had a mean of 1.016 +/- 0.133 (SEM) with a 95% CI of 0.72-1.302. The p value for this analysis was 0.016 (significance being $p<0.02$) implying that the two groups represented two separate entities. Patient #15 of group II had a mean 1:2 signal ratio of 2.14, which was the highest value for this group. When the raw data is viewed, it is evident that there were two problems with this analysis, firstly that the percentage of cells with hybridised signal was uniformly low and that one high signal ratio of five, skewed the analysis of this patient, therefore it is difficult to accurately assess the deletion status of this patient with the present data. No further sample was available to repeat the hybridisation of MRP to this patient's nuclei. The exclusion of this patient gave Group I a mean of 0.935 +/- 0.114 with a 95% CI of 0.688-1.183 which is comparable to the control group, however, the p value for the comparison of the two groups does not significantly change.

The *MRP* expression data was analysed using the log of the expression ratio to normalise the data and applying a Student's t test. The *MRP* deletion group was again designated Group I and the non-deletion group was designated Group II. For Group I, the mean value was 0.094

with a standard error (SEM) of 1.722, the 95% confidence interval (CI) being 0.017-0.53. Group II had a mean value of 2.41+/- 1.445 (SEM). The 95% confidence interval was 1.37 - 7.33. The Student's t test of unequal variance produced a p value of 0.003 with $p<0.005$ being of significance for this data. This implied that the two groups: deleted vs non-deleted vary significantly from each other in terms of *MRP* expression. Analysis of this data using a Wilcoxon's rank sum test gives a one-sided p-value of 0.0016, when testing for lower *MRP/TFRR* ratios in the "deleted" group (Group I).

Very limited data was available concerning patient outcome, in particular the duration of first remission or time to failure (with failure being defined as time to relapse from first remission or death) was not available. The analysis of the survival data using a Wilcoxon's rank sum test of significance in terms of absolute survival irrespective of remission status vs. *MRP* expression ratio gives a p value of 0.42 (based on exact calculations using StatXact Turbo). The median survival from the Kaplan-Meier estimates of the survival distributions for the group in which the MRP/TFRR expression ratio was >3 was 11 months however the median survival for the group in which the MRP/TFRR ratio was <3 has not been reached. When the dichotomy is chosen on the basis of MRP deletion vs. no deletion, no significant difference is seen between the two groups: p=1.00 (based on exact calculations using StatXact Turbo).

5.5 Discussion

5.5.1 Discussion of the Data

The data from this chapter concerned the measurement of the relative level of transcription of *MRP* and the determination of the number of MRP alleles present in leukaemic cells in the

bone marrow aspirates of a cohort of leukaemic patients carrying chromosome 16 abnormalities, in particular the inv(16). The data obtained facilitated analysis of the cohort for MRP expression, MRP deletion status and the effects these variables had on survival of the patient.

Controls for the expression analysis and the deletion status were selected on the basis of similar immunophenotype characteristics to that of the patient cohort. The CD34+ cell type was chosen as a baseline for *MRP* expression and used for direct comparison to the leukaemic population. As shown in table 5.3 the majority of the leukaemic samples were CD34 and HLA-DR positive as well as having monocytic and more differentiated myeloid characteristics. RNA was extracted from neutrophils to assess the expression of MRP in differentiated myeloid cells however insufficient RNA could be extracted for analysis. RNA extracted from monocyte preparations was sufficient for RT-PCR and revealed an *MRP/TFRR* expression ratio of 0.5, relative to CD34+ cells. This suggested that differentiated monocytes produce less transcribed *MRP* than the more primitive CD34+ cell population which includes haematopoietic stem cells. If available, a number of monocyte and CD34+ samples could be tested to establish the range of expression of these cell types. The 3 normal bone marrows examined in the current study yielded *MRP* expression ratios of consistently less than one. This was most likely due to a high proportion of differentiated myeloid cells in the preparations which may express lower levels of *MRP* than CD34+ cells, as is the case with P-glycoprotein (Drach 1992). Data concerning the expression of P-glycoprotein (Drach 1992, Chaudhary 1991) and functional drug efflux studies examining *MDR1* expression and drug efflux in CD34+ cells (Leith 1995) suggest that P-glycoprotein

expression is differentiation status dependent. CD34+ cells, unlike differentiated myeloid cells actively transport Rhodamine from the cell suggesting active P-glycoprotein and MRP activity (Feller 1995). The normal bone marrow consists of significantly less than 1% CD34+ cells, the majority of cells being myelocytes, metamyelocytes and terminally differentiated myeloid and erythroid cells as well as T lymphocytes. It has been hypothesised that the expression of MDR genes may serve the biological purpose of protecting the stem cell population from naturally occurring cytotoxic compounds (Gosland 1991). This cellular defence mechanism is less important at the differentiated cell stage and accordingly, *MDR1* for example, is expressed at much lower levels in differentiated myeloid cells (Drach 1992). From the data shown in this chapter, it would appear that *MRP* has a similar expression profile.

With regard to the other control cells used, peripheral blood mononuclear cells (PBMC) from three different sample sources, gave the highest MRP expression ratios: 2, 2, and 4. It was noted by Cole (1992) that *MRP* mRNA was readily detectable by Northern analysis of total RNA preparations in PBMCs which would predominantly consist of T lymphocytes. It was also noted in this study that *MRP* expression was undetectable in spleen extracts, which are a mixture of T and B lymphocytes. Abbaszadegan 1994 analysed a range of peripheral blood mononuclear cells using RT-PCR and found a similar basal level of expression of MRP in all cell types investigated and suggested that there was little overall variation in this ubiquitously expressed gene. This author found that CD4, CD8 and CD56 cells expressed the same basal level of MRP as CD19 cells using RT PCR within the exponential range of the reaction. The

differences between the studies by Cole and Abbaszadegan may relate to differences in the techniques employed rather than actual differences in the cell populations studied.

The human myeloid leukaemia cell line, U937, was also examined in this thesis and was selected because of its monocytoid phenotype. This drug sensitive cell line has been used for analysis of the expression of *MRP* by Slapak (1994). *MRP* mRNA was undetectable in the parent cell line by Northern blotting analysis and was only detected by that method after 10-50 passages of exposure to the anthracycline, doxorubicin. However, in this thesis, using the technique of RT-PCR, *MRP* expression was readily detectable in U937 cells. Once again the expression ratio for U937 cells was less than one, significantly less than the expression of *MRP* by PBMNCs which is readily detectable by Northern blot analysis (Cole 1992). Leukaemias known to express P-glycoprotein were also examined, as listed in table 5.4, and shown to express levels of *MRP* similar to CD34+ cells.

Considering the MRP deletion status analysis, the cohort can be divided into two groups and the expression of MRP analysed for each group. Group I in which a deletion of one *MRP* allele had occurred in relation to the inv(16) and Group II in which no such deletion had occurred. Within Group I, the mean of the *MRP* expression values was significantly lower than the second group ($p=0.003$) and all values for *MRP* expression in the deleted group were lower than the level of expression in the control cells (CD34+ flow sorted haematopoietic precursors). It was hypothesised that the reduced level of expression seen in the *MRP* deletion group is due to the absence of one *MRP* gene on the chromosome with the inv(16) and suggests that there has been no compensatory up-regulation of the remaining gene on the

normal chromosome 16. This would imply a dosage effect with the number of genes present being related to the relative quantity of mRNA transcribed and therefore to translation of protein (Grant 1994). However, in the analysis of the *MRP* expression data, although the mean *MRP/TFRR* ratio for each group is statistically different, there is an overlapping range of expression. Within Group II in which there is no deletion of *MRP*, there exists levels of expression that are as low as the *MRP* deletion group ie patient #17 (0.4) and patient #25 (0.5). This may be due to down regulation of expression of the MRP gene on the inv(16) due to the loss or disruption of transcriptional regulatory elements upstream from *MRP*. The promoter region for *MRP* has been cloned and found to lie in a 9kb genomic DNA fragment and represents nucleotides -91 to +103 in a GC rich region of the *MRP* cDNA (Zhu 1994). The distance separating the breakpoint cluster region of the short arm of the inv(16) and the 5′ region of the *MRP* gene is approximately 150kb, the 5′ end of *MRP* being situated closest to the *MYH11* short arm breakpoint as was established in chapter 4 of this thesis (figure 4.9). However, little is known of the mechanisms of regulation of *MRP*, with sequence analysis of a 2.2kb PstI fragment containing promoter activity for *MRP* indicating the presence of AP-1, AP-2, SP-1, ERE, GRE and CRE consensus domains (Zhu 1994). The author described positive and negative regulatory elements for *MRP*. In the study by Zhu, it was found that a fragment which extended 5′ from the promoter region by 318 nucleotides was devoid of promoter activity yet a fragment extending to 91 nucleotides 5′ from the promoter region upregulated the expression of *MRP*. This indicated that the former region contained an element which down regulated *MRP* and it was found that this region contained the sequence AACCTCT, which is characteristic of the NE-1 negative regulator found in a number of

genes (Larsen 1990). It was suggested in this study that the response of *MRP* transcription to these elements may be tissue specific.

The specific effects of the alteration of the tertiary structure of the DNA 5′ from the promoter region of the *MRP* gene following the inv(16) event is not known and it may be that regulatory elements for *MRP* may be situated some distance from the transcriptional start sites of *MRP* as is the case for the locus control region for β globin genes (Bresnick 1994). Patient #25 who demonstrated low expression of *MRP* without evidence of a deletion of an *MRP* allele, was shown to have the common breakpoint transcript and is not known to have any unusual aspects to the morphology of the translocated chromosomes. Patient #17 also has a low level of expression of MRP without evidence of a deletion of the MRP gene on the inv(16) chromosome. In this patient the *CBFB/MYH11* fusion transcript type is not known. It may be that in these patients, the coding region of the MRP gene was not deleted, however upstream regulatory elements may be involved in a small but significant deletion associated with the translocation event. It is in these patients that the additional information hoped to be obtained from the Southern analysis of restricted patient DNA, using *MYH11* probes situated within 10kb of the short arm breakpoint of the inv(16) and t(16;16) chromosomes may have valuable. The data from the Southern analysis may have revealed an association with small inv(16) and t(16;16) associated deletions and the level of expression of *MRP*. Further knowledge of the regulation of *MRP* and the genomic structure of the regulatory elements for *MRP* is required to assess the effects that the specific inv(16) breakpoint for each patient has on *MRP* expression.

In a study by Eijdems (1995), a number of lung cancer-derived MDR cell lines, that covered a range of drug resistance levels were studied and shown to over-express *MRP*. They described a 3 fold increase in *MRP* expression in the cell line 30.3M (following exposure to anthracyclines), over the parent non-small-cell lung cancer cell line SW-1573, as causing weak drug resistance, not associated with gene amplification. Analysis of the weakly drug resistant cell line showed that the increased expression was due to transcriptional activation of the gene. The mechanism for increased drug resistance induced *in vitro* in cell lines following multiple exposures to high levels of chemotherapeutic drugs, appears to be amplification of the *MRP* gene (Eijdems 1995, Cole 1992). In Eijdems' study, a strongly drug resistant lung cancer cell line, GLC4/ADR, with marked over-expression of *MRP,* was associated with amplified *MRP* sequences present in double minutes and homogeneously staining regions on chromosome 16. However, gene amplification as a mechanism of drug resistance *in vivo* in human leukaemias has not been demonstrated. The increase in expression of *MRP* and other MDR related genes *in vivo* is more likely to be due to the upregulation of the gene, as found in Eijdems' study of low level resistant cell lines. In studies where *MRP* expression has been semi-quantitated, low level resistance has been documented with a 2.1 fold increase in *MRP* expression above the parent cell line K562 following exposure to cytotoxics (Zhou 1996). At this low level of resistance, amplification of the gene was not documented.

Another interesting aspect of the *MRP* expression data from this chapter, was the markedly increased *MRP* expression seen in some patients within Group II. Patient #29 (MRP expression ratio 4) had secondary AML following cytotoxic treatment for breast cancer and

this may in part account for the increased expression of *MRP*. In this patient, and patient #27 (*MRP* expression ratio of 10) both have diagnoses of del(16q22) leukaemia which are known to have a poorer prognosis compared with patients with inv(16) and t(16;16) leukaemias. Patients #1, #23 and #27 were not previously exposed to chemotherapy and patient #14 (expression ratio 5) had otherwise no poor prognostic features however died 150 days after diagnosis and commencement of induction chemotherapy. Based on the observations of Zhou and Eijdems, high expression of MRP may be taken as an *MRP* ratio of greater than or equal to three. If this cut off point is applied to the current study, it is observed that all of the patients with a ratio of *MRP* expression greater than 3 are deceased. However, at this time, the survival of this group of patients does not differ significantly from the group containing all other patients in whom the expression ratio of *MRP* was known to be less than 3. Patient #7 (expression ratio 0.5), patient #12 (expression ratio 0.02) and patient #17 (expression ratio 0.4) were all alive at the period of last follow up but their survival duration was low at that time. The follow up data for these patients are already one year out of date and it would be interesting to obtain up to date data on these three patients for re-analysis. This is because all patients with very high MRP ratios (>3) were already deceased at the time of ascertaining the survival data for the thesis and therefore will not alter on review. Unfortunately no further survival data will be made available until July 1997. When the study code is broken on SWOG study 9034, other aspects of response to chemotherapy may also be evaluated.

It is most likely that there is a correlation between the level of expression of the *MRP* mRNA and protein, and the functional effects of the MRP protein (Grant 1994). In a study by Ross (1996), a quantitative RT-PCR assay was used to measure *MRP* expression in leukaemic

blasts of AML patients. The level of expression was compared to HL60 cells and was found to be up to 1.7 fold increased over this baseline which correlated with a functional defect in the transport of daunorubicin. However it has not been determined if there is a specific threshold level of expression of *MRP* above which the drug resistant phenotype is clinically manifested by the leukaemia. Neither a continuum nor a threshold theory have clearly been established as fact but from the current study it has been observed that in patient #22 where the *MRP* expression ratio is 2.5 the patient remains alive at 1134 days whereas all of the patients classified as high *MRP* expressers, with ratios of 4-10, were deceased long before that time, the longest surviving member of the group being patient #1 who died at 405 days. However, the analysis of larger numbers of patients is required to statistically evaluate these observations. One difficulty with the analysis of clinical data as opposed to *in vitro* data is that not all variables are comparable from patient to patient therefore it is difficult to confidently exclude the possibility of mitigating factors in the clinical outcomes of the patients. This problem is attempted to be overcome by prospective controlled clinical trials which must be well planned using up to date knowledge of the biological aspects of the disease and be well instituted to ensure that all patients receive the same high standard of care. Despite this, the current study demonstrates that the inv(16), and the t(16;16) leukaemias are a non-homogeneous group in terms of *MRP* expression and the response of the leukaemia to chemotherapy. It identifies for the first time, that deletion of the *MRP* gene results in lower overall *MRP* expression and suggests that not only the deletion of *MRP* but also the associated inv(16) event may play a role in the reduced expression of *MRP*. The data from the literature and findings from this study suggest that the reduced *MRP* expression may result in lower intrinsic drug resistance of the leukaemia at the time of presentation. These

findings suggest that *MRP* and probably other MDR genes should be quantitatively assessed prior to induction chemotherapy so that decisions regarding the clinical management of patients regarding for example, early vs. late transplantation may be made with some greater insight into the biology of the leukaemic disease for each patient.

5.5.2 Concluding Remarks

A major difficulty faced in this section of the thesis work was that of the poor viability of the cryopreserved bone marrow samples. This was most likely due to their transport on dry ice from New Mexico to Canada and then to Australia. This prevented a number of planned experiments from being performed with success. A considerable amount of time was invested in the setting up of short term cultures using growth factors for the harvesting of metaphase chromosomes, for analysis of the *MRP* deletion status of the leukaemic samples. This method I believe is the "gold standard" for the analysis of gene deletion and is superior to gene dosage analysis by Southern and to interphase analysis as chromosome morphology can be examined. The leukaemic samples after short term cultures were uniformly dead by the time of harvest. A second experiment planned was that of FACS (Fluorescence Activated Cell Sorting) analysis of the leukaemic cells using monoclonal antibodies against *MRP*, obtained from Dr Susan Cole. This would have provided much needed data on the correlation of *MRP* gene expression and protein expression. Unfortunately the cryopreserved cell analysis resulted in a large amount of non-specific binding, presumably due to increased cell death, which could not be gated out and the FACS analysis had to be abandoned.

The poor cell viability also restricted the number of samples which could be analysed by RT-PCR and resulted in probable degradation of large size DNA. In virtually all patients, sufficient RNA could be extracted however in some patients the RNA was of relatively poor quality and the expression of *MRP* and the *TFRR* control was too low to be analysed by the chosen technique. In the cells where results are shown, the RNA was of good quality and sufficient quantity to enable appropriate numbers of PCR experiments to be performed to obtain reliable and reproducible results. The use of fluorescence tagged primers, polyacrylamide gels and laser scanning of the products by an automated sequencing apparatus would be a more sensitive technique to use than the techniques employed in the thesis. It may be possible to re-analyse using fluorescent methods, the group of patients in whom the RNA was insufficient for analysis in the thesis and hopefully complete the data on the entire cohort. However, this was not possible within the time restraints of the thesis.

A number of methods are currently used in the evaluation of the expression of multidrug resistance genes. In the analysis of mRNA, these include Northern analysis, which is usually too insensitive an assay for use in clinical samples, slot blot hybridisation which does not truly provide quantitative information and raises other problems in terms of specificity (Zhou 1992), RNAse protection assay which is a highly sensitive and specific technique when conditions are appropriately standardised (Burger 1994, Schuurhuis 1995) and RT-PCR which is highly sensitive and specific but has potential problems related to its sensitivity and applications for quantitation (Abbaszadegan 1991, Barrand 1994, Zhao 1993, Hart 1994). The RT-PCR reaction is relatively simple in terms of the experimental requirements, particularly in comparison with the RNAse protection assay. However, problems arise due to the many amplification steps of the PCR reaction. When the cell sample is not pure, contaminating cells may spuriously affect the results leading to false elevation of the

measurement of the mRNA species, for example CD8+ and NK cells present in the measurement of MDR gene expression of haematopoietic precursors (Brophy 1994). In this study, this was addressed by the exclusion of patient samples containing more than 1-2% lymphocytes and low overall blast percentages.

The lowest percentage blast count used was that of 70% the majority being 80-95% blasts. All patient material was reviewed by me in an effort to standardise the results and generate as homogenous a population of patients as possible to eliminate some of the mitigating factors. Slides were made from the stored bone marrow sample, stained with a MGG stain and the cell populations examined. Many patients were rejected on the basis of low blast counts (ie <70%). Where patients with blast counts less than 80% blasts were used, the cell differential performed on the stored bone marrow revealed high numbers of primitive myelomonocytoid cells, obviously beyond the blast stage of differentiation but clearly part of the malignant clone and containing primitive morphological features. The immunophenotype analysis confirmed these findings largely in that high expression of monocytoid markers plus HLA-DR and CD34 were present on the analysed population, consistent with the morphological findings. Also, abnormal eosinophils were present in excess in some samples patients 11, 15, 22, 29 of which only the last two were analysed for *MRP* expression, both of which contained at least 90% blasts in the cryopreserved sample. In no cases were samples used which contained a large percentage of lymphocytes (>1-2%).

As can be seen from the control cell analysis of *MRP* expression, the expression ratio for monocytes and U937 cells is recorded as 0.5. If the results of patients with the lowest morphological percentage of blast were significantly altered by a 30% mixed population of more differentiated cells arising from the malignant clone (namely patients 1,5,14,16,27), their expression ratio values should have been brought down by the mixed cell contamination.

The *MRP* expression ratios for these patients were equal to or greater than normal CD34+ cell expression: 5, 1, 5, 1, 10 respectively. None of these were shown to be *MRP* deletion cases, therefore this will not affect the correlation of low *MRP* expression with *MRP* deletion. The exclusion of patients with significant numbers (ie >2%) of lymphocytes, proved to be an important consideration for the study.

It is possibile and likely that the blast population itself is non-homogeneous in terms of *MRP* expression. This concept underlies the theory of augmentation of drug resistance by selection of cells capable of surviving cytotoxic chemotherapy. However leukaemia is due to an expanded clone of blast cells present in the patient's bone marrow and peripheral blood. Aside from the difficulties of single cell PCR, it is more relevant to the patient to analyse the total cell population, realising that within this population there will be variation and that lurking within this population will be high expressers of multidrug resistance genes which will escape the effects of chemotherapy and ultimately lead to relapse of the patient. It is difficult and probably clinically relevant to attempt single cell RT-PCR. It's interest may lie at an experimental level to establish how many cells expressing high levels of *MRP* it takes to alter the measured value of *MRP* expression (or other MDR gene expression) and how this then correlates with duration of first CR. This line of thought is probably best investigated using a method whereby blast cells can be distinguished from other cells within the patient sample: a multiparameter (dual colour) immunofluorescence technique.

Despite the fact that RT-PCR is frequently used as a semi-quantitative method, the methods employed can differ markedly. Semi-quantitation may be attempted by maintaining the PCR reaction within the exponential range, as in Ross (1996) and Zhou (1995), or by the use of internal standards or competitors as in Xu (1996) and in the thesis study. The former technique necessitates that the amount of DNA used as a template in the preliminary control

experiment is chosen at the upper end of the range that willl be used in the assays, as amplification efficiency starts to decrease when the amount of product exceeds a certain level (Raeymaekers 1995). This latter technique, involving a mimic competitor alleviates some of the problems of standards which are external to each individual PCR reaction and does not rely on the reaction remaining in the exponential range.

The measurement of protein expression may be performed using monoclonal or polyclonal antibodies detected by immunohistochemistry techniques or far more sensitively and reproducibly by FACS analysis. However, even the use of monoclonal antibodies analysed by FACS has some pitfalls. False negative results can occur because of weak staining of the antibody, potentially due to low antigen density on the cell surface or due to sub-plasma-membrane antigens being inadequately detected. False positives may arise from lack of antigen specificity of the antibody as has been reported for C219 and C494 (Rao 1994, Georges 1990). Adequate controls in all of these detection methods are essential to exclude false positives and negatives.

Functional analysis of multidrug resistance provides complemetary information to that derived from mRNA and protein expression studies. However, whether drug efflux studies or more lengthy drug resistant in vitro assays are used, the information derived from them is alone, not as informative as combined mRNA/protein and functional studies. Functional studies alone, largely fail to delineate the cause of the multidrug resistance and the relative contributions of the different proteins responsible for the drug resistant phenotype and non concordant results of P-glycoprotein and efflux studies have been reported (Leith 1995, Willman 1996). Conversely, mRNA and protein analysis of P-glycoprotein are not always concordant (Chaudary 1991, List 1991). Currently, although many people believe that functional assessment of drug resistance is essential, these studies done in isolation are

themselves inadequate. At the present time, despite the use of resistance modifying agents in some protocols, few protocols for the treatment of acute myeloid leukaemia include drug resistance assesment in a prospective fashion and none to my knowledge have been reported where drug resistance assays have been used to change therapy.

The exact role that MDR plays in the outcomes of patients with leukaemia is uncertain with striking development of a drug resistant phenotype in some patients and an absence in others. Survival data correlated to specific expression of known MDR factors is largely inconclusive and incomplete. This may be because drug resistance factors significantly vary in levels of expression from one malignant cell type to another and within one cell type as well. It is likely that clinical multidrug resistant phenotypes result from the interaction of the various mechanisms of drug resistance, controlled by a variety of genes and each of these mechanisms will need to be evaluated both in expression and function, to fully understand the biology of the disease. By analysing the contribution of each of the multidrug resistance genes we may begin to gain a clearer understanding of the relationship of each gene to the multidrug resistance phenotype and clinical outcome of the patient.

The data obtained from the experimental methods used in this chapter provides new and unique information concerning the inv(16) and t(16;16) leukaemias and also provides some insight into one of the mechanisms through which the inv(16) and t(16;16) leukaemias may have a superior prognosis to that of the del(16q22) leukaemias. The information obtained in this chapter is complimentary to the information obtained from chapter 4 of this thesis, although it does not confirm or refute the findings of the clinical study within chapter 4. The outcomes of the two groups were however measured in different ways and it will be interesting to compare duration of first remission/time to failure in the patients from this chapter with the cohort used in chapter 4, once this data becomes available. The finding

when the two cohorts of patients were compared is that the significant difference in time to failure of the *MRP* deletion group of study I, chapter 4, compared with the non-deletion group of that study may be that the non-deleted group contained a large percentage of high *MRP* expressers in. This result is anticipated from the data from chapter 5 which suggests that high expression of *MRP* may confer poor prognosis to the leukaemia. The *MRP* deleted group from the cohort in chapter 5 all expressed low levels of *MRP*, however the survival difference was not shown to be significant when compared with the non-deletion group.

It is interesting to note that the expression of *MRP* and therefore the level of its transcription, in those patients in whom there was a deletion of one MRP gene (patients #2, #12 and #18) was much lower than 50% of the expression seen in CD34 cells, as might be expected with a 50% reduction in gene complement. The explanation of this is uncertain, however, as the regulatory mechanisms dictating the expression of *MRP* are incompletely understood, there may be factors present which are contributing to the down regulation of the remaining gene. It is impossible to say from the current data whether this down regulation of the second gene is an inv(16) associated event or related to some other aspect of the particular leukaemia experienced by those patients. However, the mechanisms by which *MRP* expression may be downregulated would seem an important area of investigation, speculating that it may be possible to design treatments to downregulate controlling regions of *MRP*. It is evident from this study and others that *de novo* leukaemias express *MRP* and that secondary leukaemias express greater amounts of *MRP* (Schuurhuis 1994) presumably due to upregulation of *MRP* transcription as suggested by Eijdems (1995). It is now necessary to focus on the reversal of drug resistance through functional reversing agents such as verapamil and cyclosporin and transcriptional negative regulatory factors, to maximise the effects of cytotoxic therapy, as it is at the initial presentation of the cancer that we have the greatest chance of curing the disease.

ARA, A NEW & NOVEL ABC TRANSPORTER GENE, IS ALSO DELETED IN INV(16) LEUKAEMIA

Chapter 6

6.1 Introduction

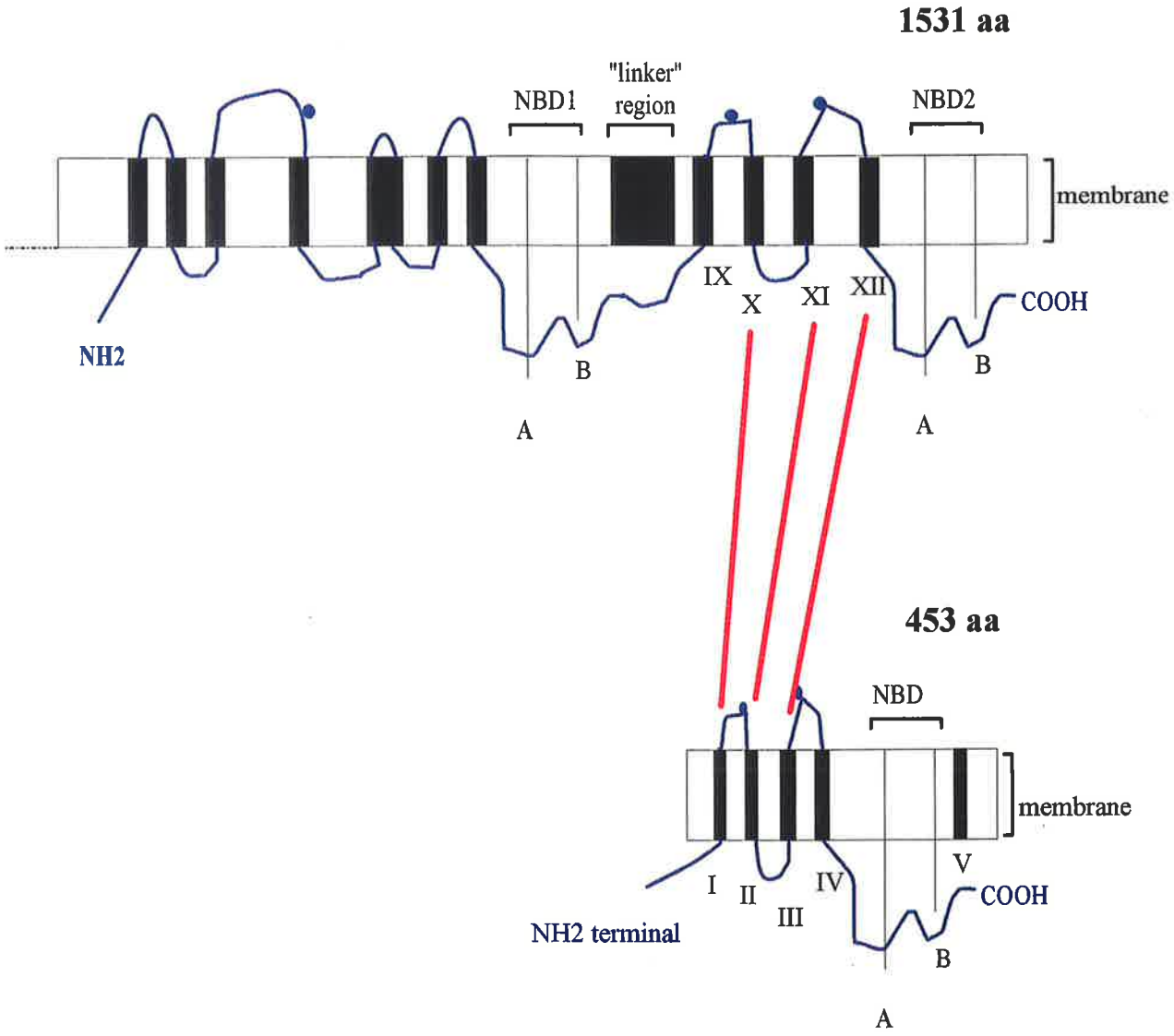
ARA is a new ATP binding cassette transporter gene which potentially encodes for a novel protein whose greatest homology lies with MRP: 51% amino acid sequence homology (Longhurst 1996). The mRNA for this gene was cloned from the human leukaemia T-cell line CCRF-CEM/E1000 after exposure of the cells to epirubicin for 18 hours prior to the extraction of total RNA. This resulted in a 2.2kb cDNA clone with an open reading frame of 453 codons and a potential protein of predicted molecular mass of 49.5kDa. This compares with a 6.6kb cDNA for *MRP* and a 190kDa protein (Cole 1992). The difference in size between the MRP protein and ARA putative protein is due to ARA potentially containing only one ATP binding site and only 6 transmembrane regions compared with two ATP binding sites and 12* transmembrane regions for MRP and P glycoprotein (figure 6.1). (* Recent data presented by Hipfner 1996 suggests 16-18 transmembrane regions)

A computer alignment of the predicted amino acid sequence of these two genes is shown in figure 6.2. *ARA* shows highest homology to the C terminal half of MRP, however there are specific regions of high homology. For example, the region centred around the Walker A site (Walker 1982) has 17 identical amino acid residues and the region around the Walker B (Walker 1982) and the ABC signature (Higgins 1992a) sites have 30 out of 35 identical residues with 4 of the 5 mismatches being conserved substitutions. The presence of the highly conserved nucleotide binding site and the similarity of the ABC signature sites of *ARA* and MRP, indicate that an ARA protein may be a member of the ABC transporter superfamily. From the predicted topology (figure 6.1) it is anticipated that ARA site I corresponds to MRP site X, ARA site II corresponding to MRP site XI and the third ARA site (III) corresponding to MRP XII. Site III in ARA is situated closer to the preceding site than site XII is to site XI of MRP, as there is an additional 67 amino acids in the sequence of MRP (figure 6.2). ARA has potentially two extra regions, ARA site IV and V. From the current knowledge of the function of ABC transporters,

Figure 6.1 Predicted topological configuration of ARA and MRP

The protein structure of ARA (453 amino acids) is compared with that of MRP (1531 amino acids) and demonstrated graphically. ARA contains a single nucleotide binding domain (NBD) with characteristic Walker A (A) and Walker B (B) sites. It has five transmembrane segments (I-V) compared with twelve transmembrane segments for MRP (I-XII). Transmembrane segments IV and V in ARA are unique. The transmembrane segments homologous to both proteins are: I, II and III for ARA and X, XI and XII for MRP respectively. ARA does not possess a linker region, as it is a single ATP binding cassette protein. NH2 denotes the amino terminal of each protein and COOH denotes the carboxy terminal for each protein. (Longhurst 1996).

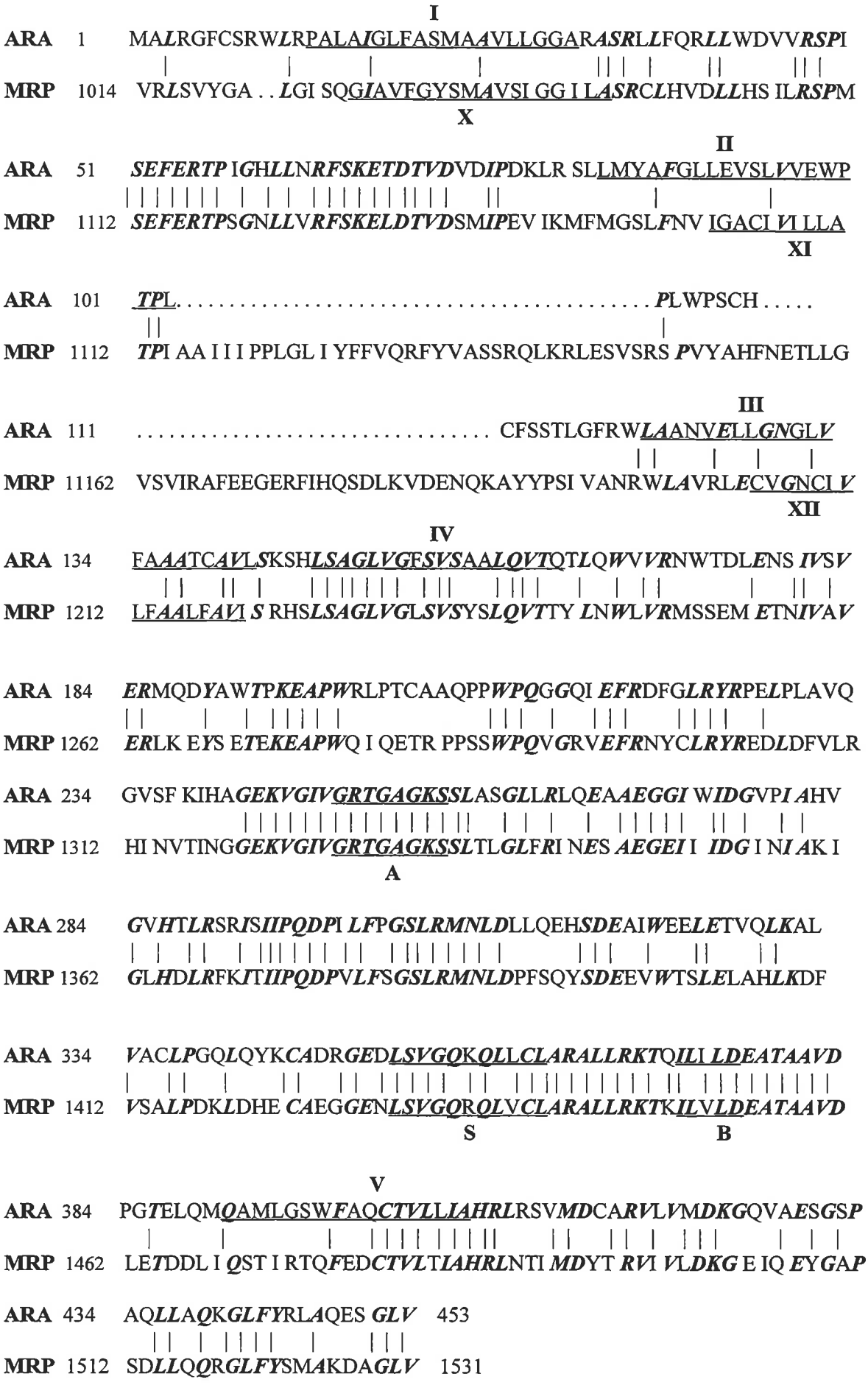
Multidrug Resistance Protein



Anthracycline Resistance Associated Protein

Figure 6.2 Amino acid sequence comparison of ARA and MRP

Roman numerals denote the transmembrane segments of MRP and ARA, the amino acid sequences of which are underlined. Homologous sequences are shown in bold face italics. Arabic numbers denote the amino acid number for each sequence at the beginning of each line. The computer analysis was taken from Longhurst (1996).


I
ARA 1 MALRGFCSRWLRPALAIGLFASMAAVLLGGARASRLLFQRLLWDVVRSPI
MRP 1014 VRLSVYGA . . LGI SQGIAVFGYSMAVSI GG I LASRCLHVDLLHS ILRSPM
X
II
ARA 51 SEFERTP IGHLLNRFSKETDTVDVDIPDKLR SLLMYAFGLLEVSLVVEWP
MRP 1112 SEFERTPSGNLLVRFSKELDTVDSMIPEV IKMFMGSLFNV IGACI VI LLA
XI
ARA 101 TPL. . . . . PLWPSCH . . . . .
MRP 1112 TPI AA I I I PPLGL I YFFVQRFYVASSRQLKRLESVSRS PVYAHFNETLLG
III
ARA 111 . . . . . CFSSTLGFRWLAANVELLGNGLV
MRP 11162 VSVIRAFEEGERFIHQSDLKVDENQKAYYPSI VANRWLAVRLECVGNCI V
XII
IV
ARA 134 FAAATCAVLSKSHLSAGLVGFSVSAALQVTQTLQWVVRNWTDLENS IVSV
MRP 1212 LFAALFAVI S RHSLSAGLVGLSVSYSLQVTTY LNWLVRMSSEM ETNIVAV
ARA 184 ERMQDYAWTPKEAPWRLPTCAAQPPWPQGGQI EFRDFGLRYRPELPLAVQ
MRP 1262 ERLK EYS ETEKEAPWQ I QETR PPSSWPQVGRVEFRNYCLRYREDLDFVLR
ARA 234 GVSF KIHAGEKVGIVGRTGAGKSSLASGLLRLQEAAEGGI WIDGVPI AHV
MRP 1312 HI NVTINGGEKVGIVGRTGAGKSSLTLGLFRI NES AEGEI I IDG I NI AK I
A
ARA 284 GVHTLRSRISIIPQDPI LFPGSLRMNLDLLQEHSDEAIWEELETVQLKAL
MRP 1362 GLHDLRFKITIIPQDPVLFSGSLRMNLDPFSQYSDEEVWTSLELAHLKDF
ARA 334 VACLPGQLQYKCADRGEDLSVGQKQLLCLARALLRKTQILI LDEATAAVD
MRP 1412 VSALPDKLDHE CAEGGENLSVGQRQLVCLARALLRKTKILVLDEATAAVD
S
B
V
ARA 384 PGTELQMQAMLGSWFAQCTVLLIAHRLRSVMDCARVLVMDKGQVAESGSP
MRP 1462 LETDDL I QST I RTQFEDCTVLTIAHRLNTI MDYT RVI VLDKG E IQ EYGAP
ARA 434 AQLLAQKGLFYRLAQES GLV 453
MRP 1512 SDLLQQRGLFYSMAKDAGLV 1531

it is expected that the putative ARA protein would need to form a homodimer or heterodimer with one of the other transporters for example, MRP.

The breakpoints for the inv(16) leukaemias were cloned by Liu (1993). Chapter 4 of this thesis demonstrated in a subset of inv(16) patients, a deletion of the short arm of the chromosome 16 is associated with the inv(16) event and this results in deletion of the *MRP* gene. It was also found that the deletion of MRP was associated with a longer time to failure as defined by death or relapse from first remission (Kuss 1994). This finding suggested that drug resistance may play a primary role in the response of the inv(16) leukaemias to chemotherapy. A study by Marlton (1995) did not find a correlation between patient outcome and a deletion of the short arm of chromosome 16 associated with the inv(16), however an *MRP* probe was not used and as stated by the author, the treatment regimens used in the study may have had mitigating effects on patient outcome. Davey (1995), reported the expression of MRP in the leukaemia MDR line CCRF-CEM/E1000 (E1000) and subsequently noted the coordinate expression of ARA with MRP. This suggests that there may be co-regulatory elements for the expression of the two ABC transporter genes following exposure of the E1000 cell line to anthracyclines. This may or may not imply close physical association of the two genes in the human genome.

6.2 Aims of this chapter

The hypotheses for this chapter were that there exists a physical association of this new ABC transporter gene, *ARA*, with its closest superfamily member, *MRP*. If these two genes are in close proximity then they may be co-deleted in a subset of the inv(16) leukaemic patients. It was also hypothesised that the range of cells, in particular haematopoietic precursors, in which *ARA* is expressed, may be similar to that of *MRP*.

The aims were to physically map the *ARA* gene using both FISH to metaphase chromosome spreads and by utilising the high resolution somatic cell hybrid panel for chromosome 16, (Callen 1995). In addition, to assess the expression of *ARA* in a range of haematopoietic precursors using RT-PCR and to compare this with *MRP* expression.

6.3 Materials and Methods

6.3.1 inv(16) leukaemic samples.

Cryopreserved bone marrow aspirates taken at diagnosis were morphologically classified confirming acute myeloid leukaemia according to FAB classification criteria and were karyotyped by routine procedures confirming the presence of the inversion chromosome 16. Table 6.1 provides the karyotypes at presentation. All samples were from Australian sources (patients #1, #4 & #5 were from the Women's and Children's Hospital, North Adelaide, South Australia; patient #3 was from the Royal Adelaide Hospital, South Australia and patient #2 was from St. Vincent's Hospital, Melbourne, Victoria) and were stored in liquid nitrogen until required for analysis. Ethics approval was obtained from the institutions involved in this study. The patients in this analysis were used for the *MRP* deletion analysis of chapter 4. Pt#1(table 6.1) = Pt#2 (table 4.1); Pt#2 (table 6.1) = Pt#20 (table 4.1); Pt#3 (table 6.1) = Pt#3 (table 4.1); Pt#4 (table 6.1) = Pt#1 (table 4.1); Pt#5 (table 6.1) = Pt#22 (table 4.1)

6.3.2 Somatic cell hybrids.

A high resolution mouse/human somatic cell hybrid panel for chromosome 16 was constructed in the Dept. of Cytogenetics at the Women's and Children's Hospital, Adelaide from patients with rearrangements of chromosome 16, as per techniques described (Callen 1995). CY18 is a somatic cell hybrid containing a normal chromosome 16 as the only human chromosome. The human chromosome 16 breakpoints contained in CY19 and CY185

Table 6.1 Clinical details and *MRP/ARA* deletion status of inv(16) leukaemic patients

Patient	**Karyotype**	**WCC** ($x10^9/L$)	**FAB subclass**	**Gene Deletion Status**	
				MRP	**ARA**
1 (16,M)	46,XY,inv(16)	104	M4Eo	deleted	deleted
2 (14,M)	46,XY,inv(16)	60	M4Eo	deleted	deleted
3 (25,F)	46,XX,inv(16)	25	M4Eo	deleted	not deleted
4 (1,M)	46,XY,inv(16)	NA	M4Eo	deleted	deleted
5 (13,M)	46,XY,inv(16)	16	M4Eo	not deleted	not deleted

inv(16) implies the standard inv(16)(p13q22) seen with the M4Eo and other acute myeloid leukaemias. F = female M = male. NA = results not available. FAB refers to the French American and British classification of acute myeloid leukaemia.

and other relevant hybrids are described in table 6.2 and are the same as used in chapter 4 of this thesis for the mapping of MRP.

6.3.3 DNA probes.

MRP probes were obtained from SPC Cole and RG Deeley, Cancer Research Laboratories, Queen's University, Kingston, Ontario, Canada. *MRP*#14 is a 3.9 kb cDNA clone that spans bases 110-4080 of *MRP* mRNA. The *ARA* probe, p15GG, was provided by R. Davey and represents a 2kb cDNA pGEM11Zf(+) subclone of ARA selected from a λgt11 cDNA library prepared from E1000 cells which express ARA. A unique 700bp Sac I/Cla I restriction fragment of p15GG was used for the probing of Southern transferred DNA and was labelled with ^{32}P using a random priming kit (Amersham). The yac clones 16y6G6 and 16y6G5 were derived from flow sorted chromosomes 16 from the somatic cell hybrid CY18. These YACs and My757D7 were obtained from the Los Alamos National Laboratories, Los Alamos, New Mexico, USA.

6.3.4 Fluorescence in situ hybridisation.

The plasmid probes were nick translated with biotin-14-dATP and hybridised *in situ* at a final concentration of 20ng/μl to metaphases from five patients with the chromosome 16 inversion. Metaphases were prepared from unstimulated short term bone marrow cultures by standard procedures (Smith 1992). The fluorescence *in situ* hybridisation (FISH) method was the same as that detailed in 4.3.6.

6.3.5 Analysis of YAC clones.

YAC clones were previously mapped by STS content to the physical interval defined by the breakpoints of the hybrids CY19 and CY185 (Doggett 1995). These included clones My757D7, My980F6, My844C8, My691D7 (from the CEPH megaYAC library) (Bellann-

Table 6.2 Somatic cell hybrids used in the mapping of *ARA* to chromosome 16

Hybrid Line	Mouse Parent	Human Parent Rearrangement	Portion 16 Present	Other Human Present
CY18	A9	nil	complete 16	absent
CY19	A9	t(13;16)(q12;p13.13)	p13.13→qter	yes
CY151	A9	t(1;16)(q12;q11.2)	q11.2→qter	absent
CY158	A9	t(5;16)(q21;p11.2)	p11.2→qter	many, normal human 16
CY185	A9	t(6;16)(p21.3;p13.12)	p13.12→qter	yes
CY198	A9	t(1;16)(?q23;p13.2)	p13.3→qter	many

Chantelot 1992) and 16y6G6, 16y6G5 (from the Los Alamos flow sorted chromosome 16 YAC library) (McCormick 1993). Primers were designed from the 3′ untranslated region of ARA to enable the screening of genomic YAC clones. The sequences of the primers used are shown in table 6.3. The reaction conditions were as follows: *95°C(2.5min), 58°C(3min), 72°C(5min) for one cycle; 95°C(45sec), 58°C(1min), 72°C(1min) for 35 cycles; 72°C ten minute extension time.* The products were visualised by ethidium bromide staining after separation on a 2% agarose gel and photographed. The positive YAC clones were digest with restriction endonucleases, Southern transferred and hybridised to a labelled restriction fragment of the ARA cDNA clone, p15GG: (700bp SacI/ClaI restriction fragment) of p15GG, labelled with ^{32}P using a random priming kit (Amersham).

6.3.6 RT-PCR.

Reverse transcription polymerase chain reaction was performed on RNA from haematopoietic cells and cell lines extracted by standard techniques (see 5.3.4.1). RT-PCR was performed using 2μg of total RNA (except for one PBMNC sample where 8μg of RNA was used) in a 20μl reaction containing 1μmol/L of random hexamers with Superscript™ reverse transcriptase as per manufacturers instructions. This was diluted with 180μl of water after inactivation of the enzyme for 10 minutes at 90°C. 15μl of this 200ul reaction was then used in subsequent PCR reactions. The primer sequences for *ARA* are shown in table 6.3. Reaction conditions for the PCR using *ARA* primers were as those outlined in 5.3.4.2: *95°C(2.5min), 58°C(3min), 72°C(5min) for one cycle; 95°C(45sec), 58°C(1min), 72°C(1min) for 35 cycles; 72°C ten minute extension time.* The products were transferred to nylon membranes and hybridised at 42°C to an *ARA* specific cDNA probe, p15GG, as per the method outlined in 2.3.4.2.

Table 6.3 Oligonucleotide primers used for PCR: *ARA, MRP & TFRR*

Gene Primer	**Sequence**
MRP F **MRP R**	AGTGACCCTCTGGTCCTTAAA GAGGTAGAGAGCAAGGATGACTTGC
TFRR F **TFRR R**	GGATAAAGCGGTTCTTGGTACCAGC TGGAGGTAGCACGGAAGAAGTCTCC
ARA 1F **ARA 1R**	TGACCCGTTGGTCATCGATAG AGATTCTGATTTAAGGGCTAGCCG
ARA 2F **ARA 2R**	ACACCCATTGGTCACCTGCTA GGTCACCTGGAGGGCAGCAGAGAC

F = forward R = reverse. ARA1F/ARA1R were designed from the 3′ untranslated region of ARA and were used in the amplification of genomic DNA [nucleotides (nt.) 1543-1563 for the forward primer and nt. 1790-1813 for the reverse primer]. ARA2F/ARA2R were designed from the cDNA sequence nt. 281-301 and 581-604 and do not amplify genomic DNA. *MRP* and *TFRR* primers were as those used in 5.3.4.2.

6.4 Results

6.4.1 Mapping of ARA to the human genome.

The p15GG cDNA clone of *ARA* was hybridised to normal metaphase chromosomes as described and viewed under a fluorescence microscope. Fluorescent signal was predominantly seen at 16p13.1 as shown in figure 6.3 but was occasionally seen at 16p11 and at the short arm telomere. No signal was seen on any other human chromosome and there was little background hybridisation evident (figure 6.3). This localised the ARA gene to the region of chromosome 16 involved in the inv(16).

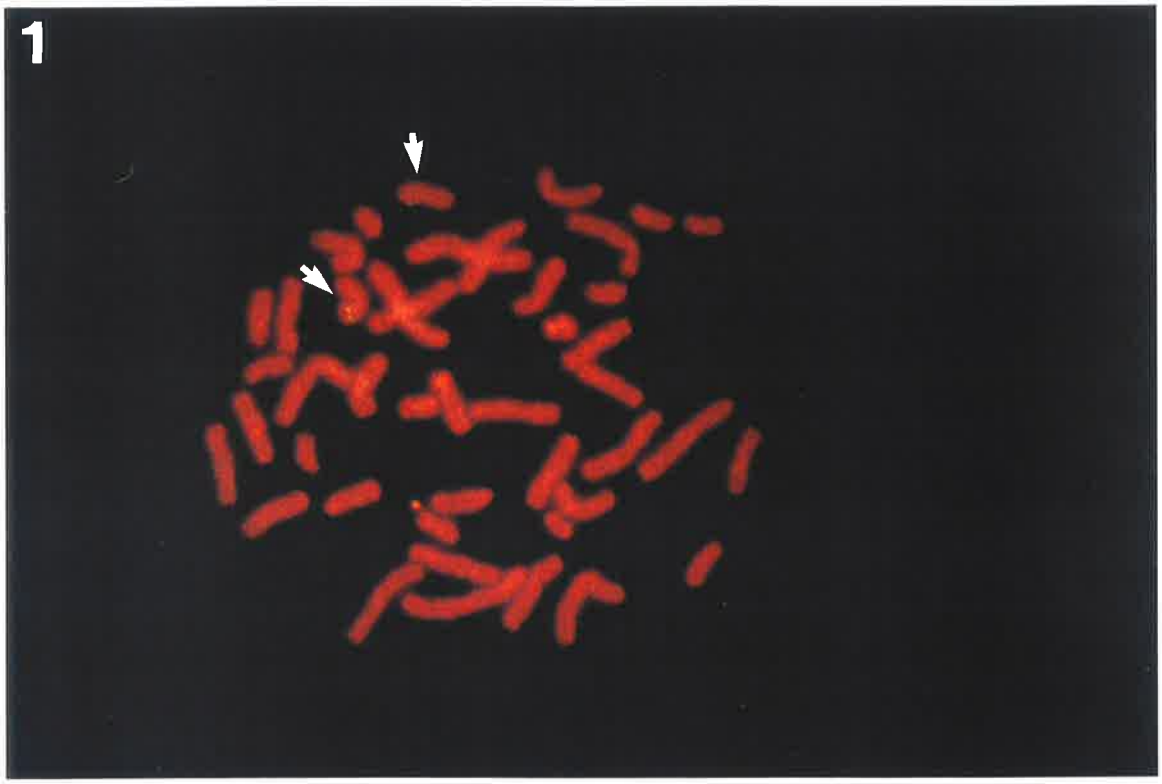
Total human genomic DNA and mouse/human somatic cell hybrid DNA restricted with the restriction endonucleases BamHI or HindIII, was Southern transferred to nylon membrane and probed with the ^{32}P labelled 700bp Cla I/Sac I restriction fragment of p15GG as shown in figure 6.4. Specific bands were seen with hybrids CY18, CY19 and CY198 (chromosome 16 content documented in table 6.2) but no hybridisation was seen with CY185 which contained only 16p13.12→qter. Corresponding bands are seen on the gel lanes containing restriction digested total human DNA. This more specifically localised the *ARA* gene to the hybrid interval defined by the somatic cell hybrids CY19-CY185, the same interval containing the short arm breakpoint of the inv(16) and the multidrug resistance gene *MRP*.

6.4.2 FISH analysis of inv(16) containing metaphases.

p15GG was hybridised to metaphase chromosomes derived from presentation bone marrows of five patients with inv(16) leukaemia. All patients had been previously studied for the presence of a deletion on the short arm of the inverted chromosome 16 using the cDNA probe, *MRP*#14. Of the five patients studied, four contained deletions of *MRP*#14 (Pts. #1, #2, #3 & #4) and one did not (Pt.#5). The gene for *ARA* was found to be deleted in three of the cases in which there was a deletion of *MRP*#14. Fluorescent signal was seen at band p13.1 in the normal

Figure 6.3 Chromosomal localisation of the ARA gene by FISH

The ARA probe p15GG was labelled with FITC and hybridised to metaphase chromosomes from a normal individual. The DAPI stain is shown to facilitate localisation of the chromosomes 16. The fluorescent signal is seen on the short arm of the propidium iodide stained chromosomes 16 (arrowed).


1

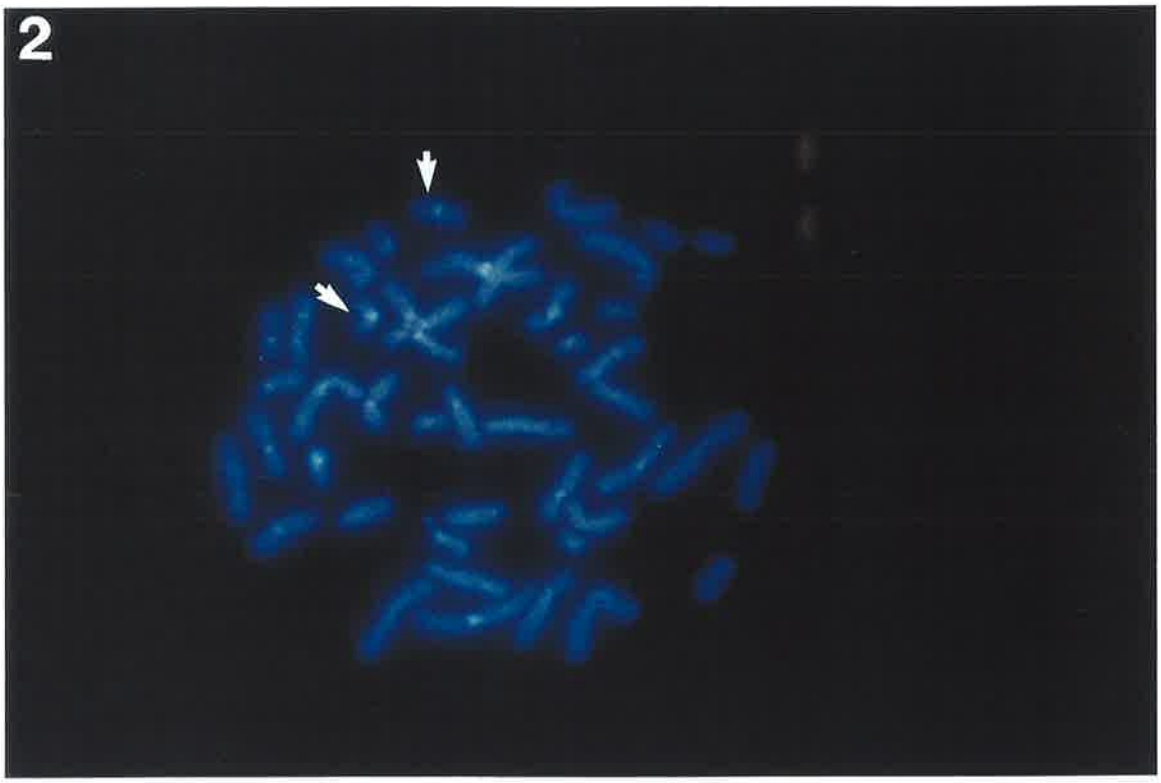
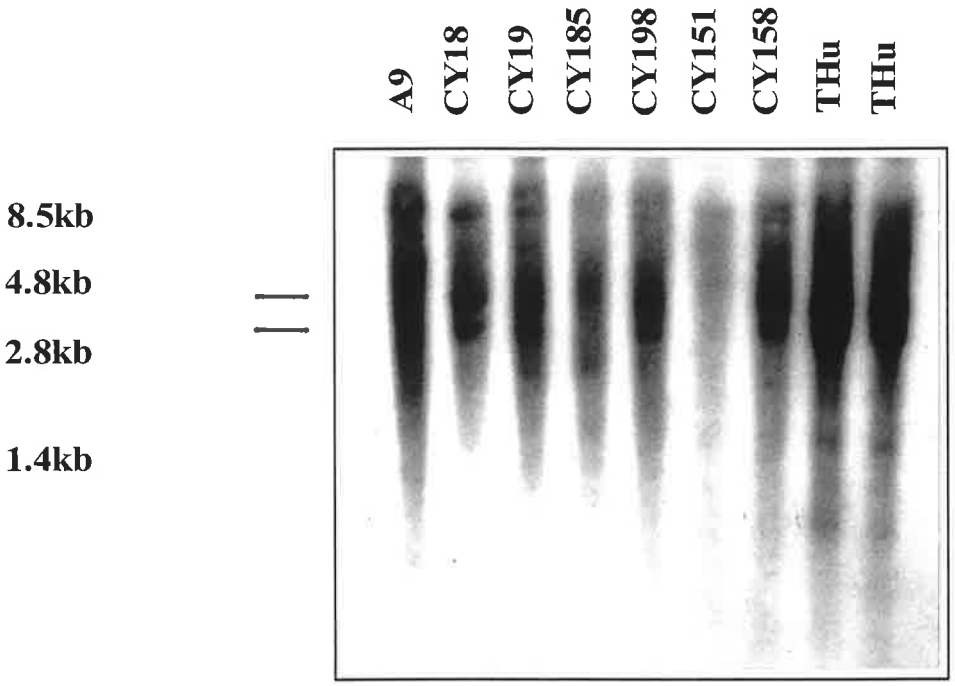

2

Figure 6.4 Localisation of ARA using Southern analysis of the somatic cell hybrid panel

The details of the somatic cell hybrids used in this Southern are outlined in table 6.2. The DNA has been digested using the restriction endonuclease BamHI. A doublet band is seen in the lanes of this autoradiograph which contain the portion of chromosome 16 positive for ARA. This analysis localises the ARA gene to the somatic cell hybrid interval defined by hybrids CY19 and CY185. THu = total human genomic DNA. Cy158 and CY18 contain intact human chromosomes 16.

Somatic Cell Hybrid Panel



Probe: 700bp ClaI/SacI restriction fragment of p15GG (*ARA*)

chromosomes 16 in the same metaphase spread as there was an absence of signal on the inv(16) chromosomes. In the patient known not to have a deletion of *MRP*#14 associated with the short arm breakpoint of inv(16), fluorescent signal was seen in the normal chromosomes 16 and the inv(16). Patient #3 was shown to be deleted for the MRP gene but not for the ARA gene on the short arm of the inv(16). Figure 6.5 shows the hybridisation of the *ARA* probe for patients #1 and #3.

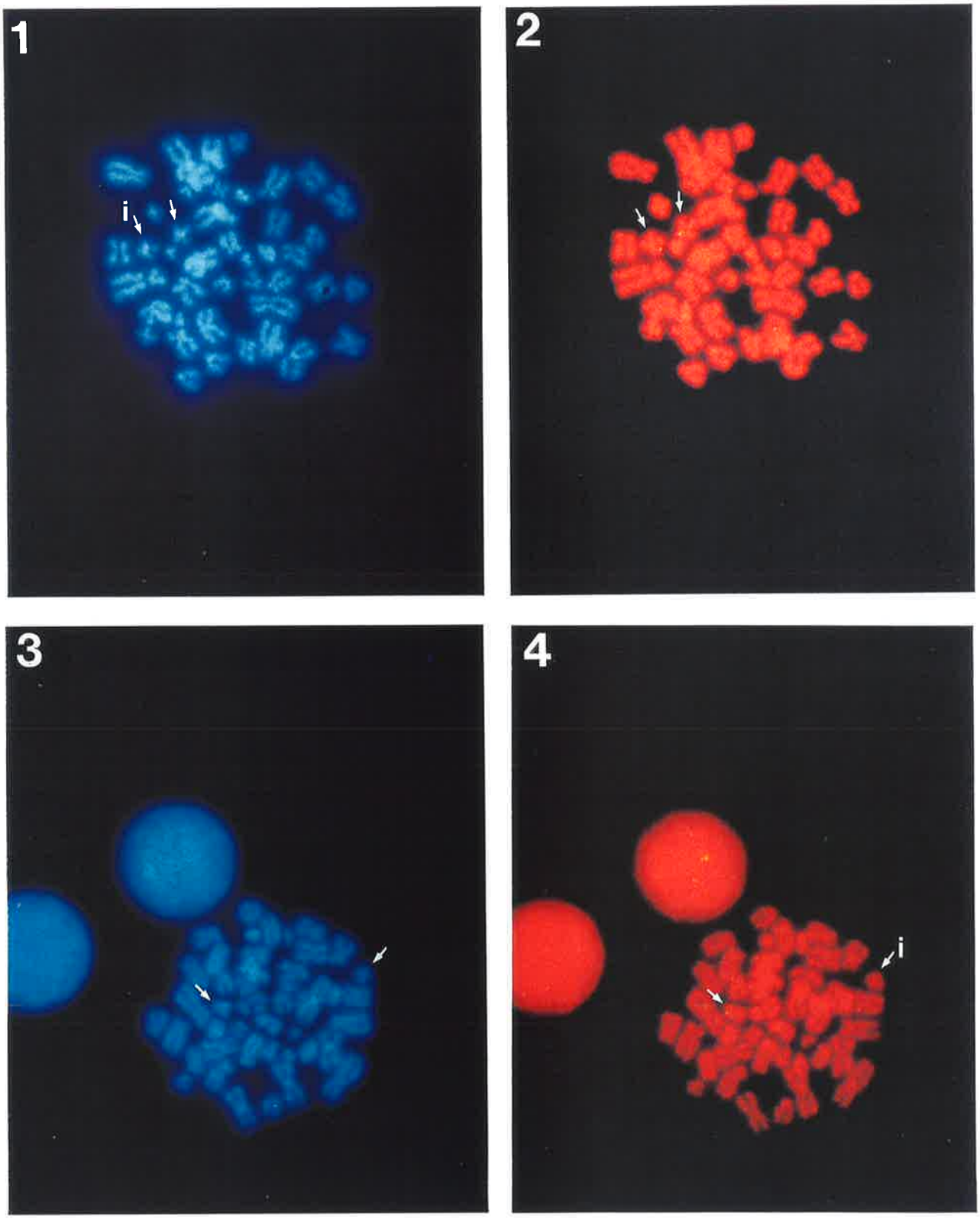
These results demonstrate that the gene for *ARA* is centromeric to the inv(16) breakpoint and therefore the *MYH11* gene. This was shown by the fluorescent signal remaining on the short arm of the inv(16), travelling with the centromere during the inversion event, as shown in figure 6.5. Patient #3, the one case in which there was a deletion of *MRP* and not of *ARA,* places the *ARA* gene centromeric to *MRP.* This is because the deletion on the short arm of chromosome 16 most likely extends as a single deletion from the primary breakpoint towards the centromere, for a variable distance for each patient. In cases #1, #2 and #4, this included *MRP* and *ARA.* However, for patient #3, the *ARA* gene was not included in the deletion, therefore, this established the gene order telomere - *MYH11, MRP, ARA* - centromere on the short arm of chromosome 16.

6.4.3 Analysis of YAC clones.

Yac clones located in the interval CY19-CY185 were obtained from Los Alamos National Laboratories, courtesy of Dr Norman Doggett. These were screened by PCR using primer pairs for *MRP* and *ARA.* The *MRP* screening was performed at Los Alamos laboratories and the *ARA* screening was performed by the student. Yac clone 16y6G5 failed to grow from the stab sent from Los Alamos and was not able to be resent in the time required to complete the thesis work. The 645kb YAC clone 16y6G6 was negative by PCR for the 3′ untranslated region of *ARA.* YAC 16y6G6 was positive however, for the more 5′ DNA sequence of *ARA* that is present in

Figure 6.5 Metaphase chromosome analysis of inv(16) leukaemic samples

Metaphase chromosomes of inv(16) leukaemic patients were hybridised with the FITC labelled *ARA* cDNA probe, p15GG. The ARA probe is shown to be present on the normal chromosome 16 but absent from the inv(16) chromosome seen in the metaphase pictured in **4.** This implies a deletion of the ARA gene has occurred in association with the short arm breakpoint of the inv(16). MRP is also deleted in this patient (patient #1 table 6.1). However, the signal for the ARA cDNA probe is present on both the inv(16) chromosome and the normal chromosome 16 in **2**. The MRP gene is known to be deleted in this patient (patient #3 table 6.1). DAPI stains are again shown to identify the heterochromatin region of chromosome 16.


1
i
2
3
4
i

the unique 760bp ClaI/SacI restriction fragment of the cDNA clone (figure 6.6). The clone My757D7 was negative for both the 3′ untranslated region of *ARA* by PCR and by Southern analysis for the more 5′ sequence included in the 760bp ClaI/SacI *ARA* restriction fragment. This 900kb megaYAC clone, which extends centromeric across the CY185 breakpoint, was also negative for *MRP* but was positive for *MYH11* (see figure 6.7). It was previously shown that a deletion within My757D7 included the *MRP* gene (Doggett 1995), and it now appears that this deletion encompasses both the *ARA* and *MRP* loci. Figure 6.7 shows the physical map for the region of *MRP* and *ARA*, as it currently is known. From the YAC localisation data reported in this chapter and chapter 4 (4.4.5), the estimated distance between *MRP* and the microsatellite marker AFM070ya1 being 75kb, the maximum distance separating *MRP* and *ARA* would be approximately 580kb.

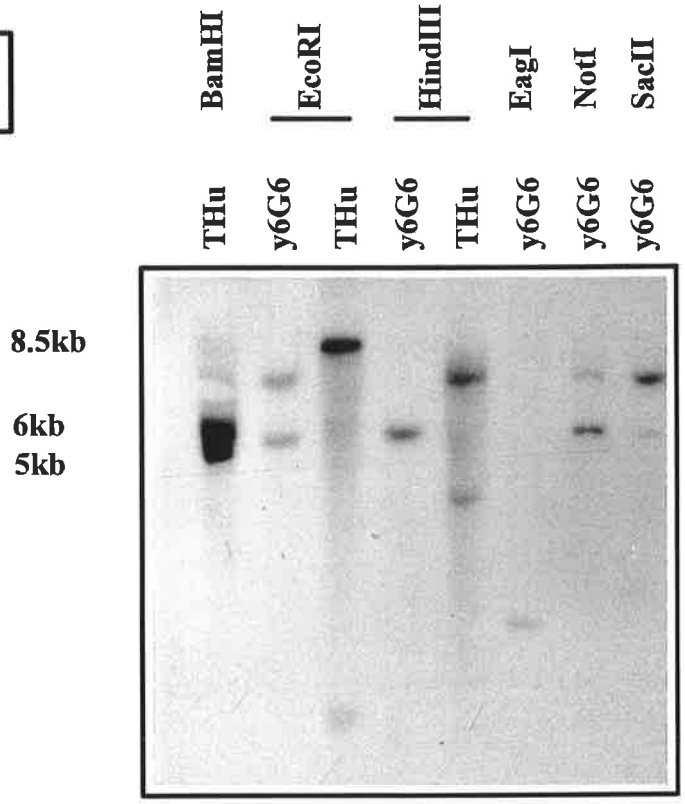
6.4.4 Expression of ARA in haematopoietic precursors and leukaemic cell lines.

RT-PCR (as per 5.3.4.1) was performed on flow sorted $CD34^+$ cells, peripheral blood lymphocytes and normal bone marrow mononuclear cells (figure 6.8). A predicted 324bp product (extending from nt. 280 to nt. 604 of *ARA)* was demonstrated in all of the cell types chosen which implies a similar range of expression for *ARA* as for *MRP*. No product was obtained using genomic DNA as the template. Transferrin receptor expression as per chapter 5 (5.3.4.2) but without the use of a competitor, was used as a positive control for the experiment (figure 6.8). The products were shown to be positive for the p15GG probe, following hybridisation of the Southern transferred PCR products to the ^{32}P labelled probe. The PBMNC sample for which 8μg of RNA was used in the reverse transcription reaction, produced several PCR products, which proved to be non specific on probing with p15GG.

Figure 6.6 Southern analysis of YAC 16y6G6

The figure represents the autoradiographic features of multiple restriction endonuclease digests of 16y6G6 and total human DNA (THu). The top figure represents hybridisation of the membrane containing YAC and human DNA with the *ARA* gene, cDNA probe, labelled with α^{32}P: p15GG. As no human bands were found to correspond with the positive YAC bands present, the membrane was stripped of hybridised probe DNA and re-hybridised with labelled pGEM vector DNA(figured below), the vector used in the construction of p15GG. This revealed that not all bands positive for the ARA gene probe were vector related and implied DNA sequence homologous to the ARA gene was present in the YAC.

Probe: ARA



Probe: pGEM (vector only)

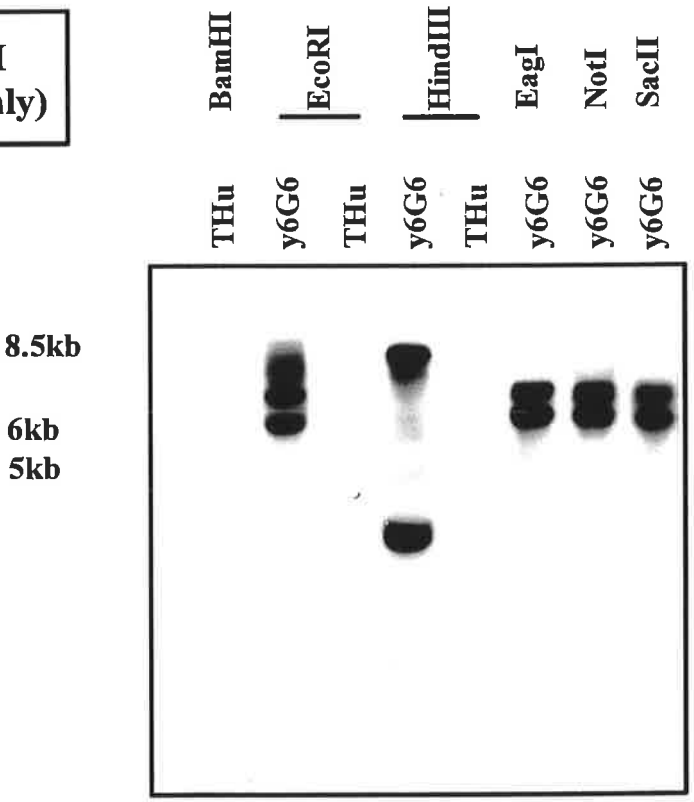


Figure 6.7 Gene and YAC map of the CY19 to CY185 interval

The relative position of *ARA* to *MRP* and *MYH11* is shown in the diagram. The diagram is not drawn to scale. The approximate distance separating *MYH11* and *MRP* is 150kb and 75kb for MRP and AFM070ya1 . The maximum distance separating *MRP* and *ARA* is 570kb. My denotes megaYAC and the prefix 16y denotes a YAC originating from a YAC library derived from a flow sorted chromosome 16. The suffix (-) implies that a deletion has occurred in the formation of this megaYAC. AFM070ya1 (*D16S*405) is the microsatellite marker demonstrated to lie between *MYH11* and *MRP* from the data in chapter 4.4.5. It is not currently known whether the YACS 6G6 or 6G5 are positive for *MYH11*. 16y6G5 is presumed to be positive for ARA from data available for 16y6G6 however this remains to be proven.

Gene and YAC Map adjacent the inv(16) p arm Breakpoint

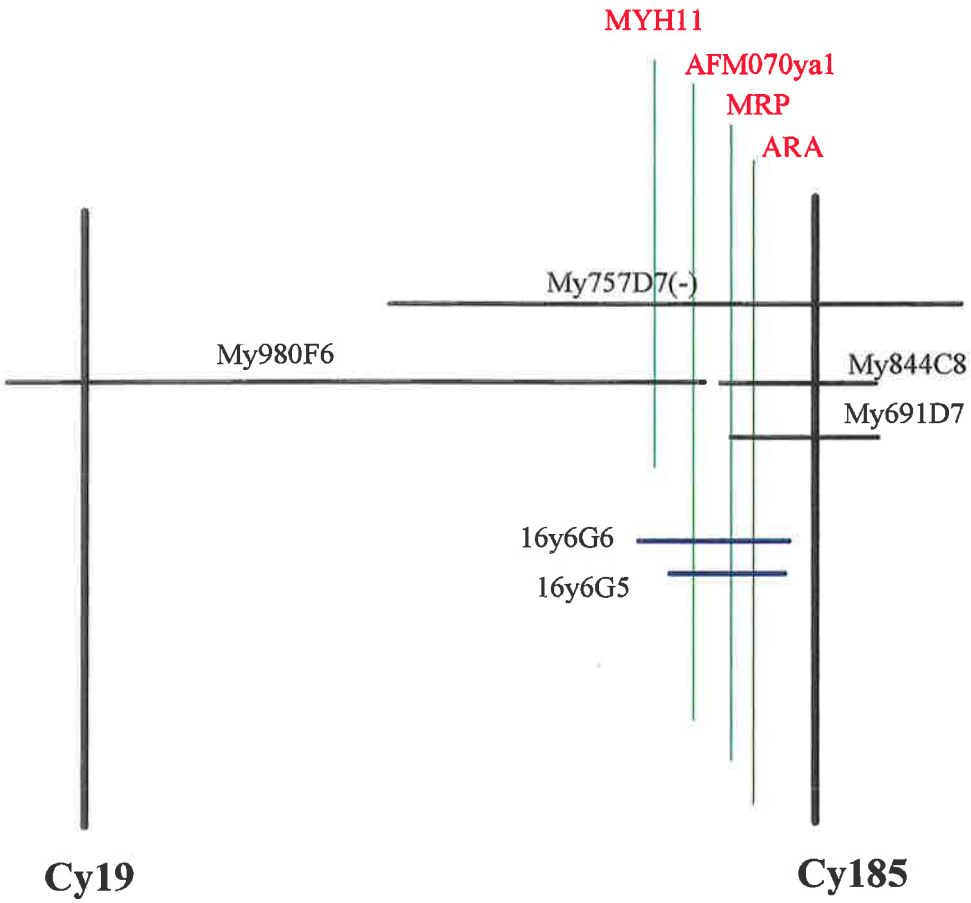


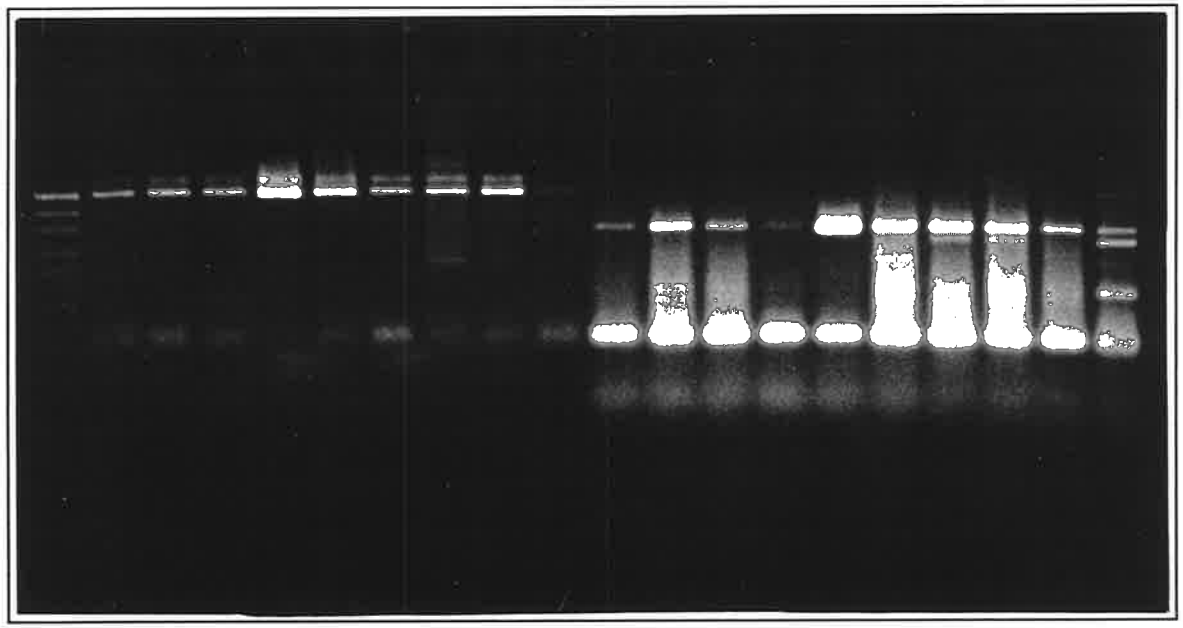
Figure 6.8 Expression of *ARA* in normal haematopoietic precursors

The expression of the *ARA* gene and the *TFRR* gene were assessed by RT-PCR. Lanes 1, 2, and 3 = normal peripheral blood mononuclear cells, Lanes 4 and 5 = CD34+ cells, Lane 6 = normal bone marrow sample, Lanes 7, 8 and 9 = peripheral blood stem cell collections, Lane 10 present only in the second half of the gel represents the expression of the *ARA* gene in a peripheral blood mononuclear cell preparation using four times the amount of target RNA for the cDNA synthesis. M = size marker: pUC19 plasmid DNA cut with the restriction endonuclease HpaII. The size markers are as follows (1/2)[shown as one band in the photograph] = 501 bp./ 484bp., (3) = 404 bp., (4) = 331bp., (5) = 242bp.

Transferrin Receptor

Anthracycline Resistance Associated protein

M 1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9 10



6.5 Discussion

The mapping of the *ARA* gene to the hybrid interval CY19-CY185, and its close proximity to both the inv(16) breakpoint and *MRP,* is interesting in two respects. Firstly, the close structural homology of the carboxy half of MRP, and the potential coding sequence of the ARA gene. In the original paper describing the MDR1 gene (Chen 1986), the authors described the gene as having an internal duplication relating to the nucleotide binding domains and the transmembrane regions. MRP is now believed to possess 16-18 transmembrane domains (Hipfner 1996) but has two nucleotide binding domains dividing the molecule into two halves with specific sequence similarity. If *MRP* were to have arisen from a duplication event from a parent gene, it may be that the *ARA* gene is a more primitive gene in evolutionary terms.

The second important point arising from the localisation of *ARA* is the deletion of an *ARA* gene in a subgroup of the inv(16) leukaemias, known to have a deletion associated with the short arm breakpoint. The deletion of MRP in a subgroup of the inv(16) leukaemias, a subtype of leukaemia known to be chemosensitive, suggested that the two observations may not be mutually exclusive. The expression of *MRP* in primitive haematopoietic precursors implies that these cells have an intrinsic drug resistance prior to any exposure to anthracyclines and vinca alkaloids. The expression of *ARA* in these cells has as yet an unknown function. It is now necessary to look at the expression of *ARA* in the inv(16) patients to determine whether there is a significant alteration in *ARA* gene expression associated with deletion of one of the two normal copies of the *ARA* gene.

MRP is transcribed from telomere to centromere(data from chapter 4) and from the data presented in this chapter, the orientation of ARA is most likely the same. 16y6G6 is negative for the 3′ end of *ARA* by PCR but positive for a 760bp restriction fragment of *ARA* encoding for more 5′ sequence of the gene by Southern techniques. This YAC clone is not known to

carry any genomic deletions. The maximum distance separating these two genes, according to the current data is 570kb, however, it is possible that the distance between the 3′ end of *MRP* and the 5′ untranslated region of *ARA* may be only a few kilobases. It will be important to follow up this initial localisation of *ARA* with more detailed analysis of the physical relationship of *ARA* and *MRP*.

At this point in time, an ARA protein has not been identified using either monoclonal antibodies directed at specific MRP unique epitopes which should be present in a putative ARA protein, nor has it been identified using polyclonal sera against MRP. Therefore, the discussion of this gene being translated into an ABC transporter is at this point speculative. The PCR primers designed for the RT-PCR reaction, fail to amplify genomic sequence, suggesting the presence of intronic sequence between these primers. Therefore it would appear that the *ARA* gene has intronic sequence making it unlikely that the sequence represents a pseudogene. A 45kDa protein has not in the past been detected by P-glycoprotein or MRP monoclonal antibodies (Flens 1994, Hipfner 1994) however a polyclonal antibody raised against the carboxy terminal 15 amino acids of MRP detected a 45kDa deglycosylated protein in the endoplasmic reticulum of MDR cells (Krishnamachery 1994). It may be necessary to develop monoclonal antibodies against ARA protein derived from eukaryotic translation of cDNA

Considering that the coding sequence predicts a high similarity of the putative protein to ABC transporters it is interesting to examine the differences that would thereby exist between an ARA protein and other ABC transporters. Most mammalian ABC transporters have two ATP binding sites, however there are a number of proteins with a single ATP binding site and associated transmembrane regions. These are all organelle associated proteins. In humans there are two peroxisomal membrane ABC transporters, PMP70 (Kamijo 1992) and ALDP (Mosser 1993) and two endoplasmic reticular ABC proteins TAP1 and TAP2 (Beck 1992). The latter

two proteins transport major histocompatibility complex class I-associated peptides (MHC-I) into the endoplasmic reticulum (ER) and their expression has been associated with MRP related multidrug resistance and with P-glycoprotein induced modulation of MHC class I antigens (Masci 1995, Izquerido 1995). The structural differences of the putative ARA protein compared to the other ABC transporters may be due to the tailoring of the gene for a specific substrate for its major biological function. There is evidence that other ABC transporters have specific functions, for example, another closely related ABC transporter, YC1 (yeast protein) is specifically involved in cadmium transport (Szczypka 1994), and recent data suggests that the biological function of MRP may be that of an ATP-dependent pump for leukotriene C_4 and structurally related amphiphilic compounds, including glutathione-S-conjugates of xenobiotics, as seen in the canalicular membrane of the liver (Leier 1994, Gekeler 1995b). In the E1000 cell line and other *MRP* expressing cell lines, partitioning of multidrug resistance associated cytotoxic agents (for example anthracyclines) into the endoplasmic reticulum has been hypothesised (Davey 1995, Marquardt 1992) and evidence has implicated conjugation of drug to glutathione prior to or as part of the transport process (Müller 1994). It may be possible that *ARA* codes for an organelle associated protein and has a role in the transport of glutathione conjugated compounds across the ER membrane, this hypothesis remains to be proven.

The finding of two ABC transporter genes within a limited region of DNA raises the possibility of a gene family of ABC transporter proteins situated on chromosome 16. Recently, another ABC transporter so-called ABC-C, has been localised to 16p13.3 (Klugbauer 1996). This gene was identified from a human medullary thyroid carcinoma cell line and is also thought to be involved in the development of resistance to xenobiotics. Slovak 1995, recently mapped the *LRP* MDR protein to 16p11.2 using dual colour FISH. However, this gene has no significant homology to the ABC transporter superfamily.

Other human derived ABC transporters have been found to be in close physical linkage, namely *MDR1* and *MDR3*. Both are situated on chromosome 7 at 7q21.1 and have been linked on a 230kb pulsed field restriction fragment (Lincke 1991). It has been found that the *MDR1* and *MDR3* genes may be amplified in parallel in drug resistant cell lines despite the fact that *MDR3* probably has no role in the development of multidrug resistance (Meese 1989). It is probable that *MDR1* and *MDR3* would be included within one amplicon following the exposure of the cell to chemotherapeutic agents as it is unlikely that there exists selective pressure to independently amplify *MDR3* from *MDR1* following exposure of cells to cytotoxic agents. Using FISH techniques on metaphase chromosome spreads, *ARA* and MRP have been shown to be co-amplified in the human T cell leukaemia cell line, CCRF-CEM/E1000 from which the ARA cDNA clones were derived (data not shown). The findings presented here are an interesting *entrez* into the investigation of a new gene in close physical association with *MRP*.

CONCLUSION

Chapter 7

Conclusion

The technologies currently available for molecular biological research allow investigation into the molecular mechanisms of the development of cancer that were only dreamed of 15 years ago. The investigation of the genetic events involved in the development of cancer have revealed that a variety of different cellular mechanism are involved in that process. The information resulting from this work has led to the development of diagnostic tests that provide more sensitive detection methods for the existence of cancer. This is particularly important in the diagnosis of minimal residual disease which is an important issue in the treatment of acute leukaemias and some forms of lymphoma. However, despite the cloning of genes involved in the cancer breakpoints and regions of loss of heterozygosity, few real inroads have been made into the development of therapies which improve survival or cure the disease. The cloning of the t(15;17) of promyelocytic leukaemia, now known to involve the retinoic acid receptor alpha, has resulted in the use of all trans retinoic acid in the induction therapy of patients with this form of leukaemia. This is unlike the *BCR/ABL* rearrangement of chronic myeloid leukaemia for which there has been no change in therapy consequent upon the knowledge of the two genes involved in the breakpoints of the t(9;22). Presently however, there are clinical trials in progress utilising antisense strands of cDNA in the treatment of haematological malignancies and there are a myriad of cellular treatments enlisting immunological manipulations *in vivo* and *ex vivo* using the knowledge obtained from molecular and cellular biological research.

The inv(16) anomaly was first described as a clinicopathological entity in 1983 by Michelle LeBeau however it has only been in the last 2 years that the actual genes involved in the inv(16) breakpoints have been cloned (Liu 1994). The data reported in chapter 3 of this thesis was aimed at the cloning of those genes however did not achieve this end. It did however serve the purpose of teaching me the molecular biological techniques necessary to complete the work for the remainder of the thesis and also provided a detailed map of the region including possible cloned genes as there was at least one CpG island present in the YAC clones received from CEPH. The cloned DNA resulting from the work for this chapter was sent on to another group working on this region of chromosome 16 with the aim of cloning genes near *CAVII*, which had been previously mapped to the hybrid interval CY4-CY130D. The mapping information resulting from the work for this chapter was forwarded on to Dr. Norman Doggett to be included in the chromosome 16 genetic data base, contributing to the work of Dr Doggett and Dr David Callen on the Chromosome 16 Human Genome Project.

The following three chapters are inter-related in terms of the hypotheses investigated and the data obtained. The biology of the inv(16) leukaemias is important to note. In general, the inv(16) and t(16;16) leukaemias have a good prognosis (Larsen 1986) but as can be seen from both of the cohorts used in this study there are patients whose outcome is poor. Of the total number of patients with inv(16) or t(16;16) leukaemia (39 patients), 9 patients were deceased by 188 days (approximately 6 months) post diagnosis which is 23% of the total. This compares with a 30-50% failed induction rate of all leukaemic subtypes for patients aged 16-60 years, most of whom survive less than 6 months (Watilin 1991). The induction rate for all patients in this thesis is not known. Most patients had the inv(16)(p13q22.1) and

t(16;16)(p13;q22.1) breakpoints. Trisomy 8 was a common occurrence in both of the studies but not found to influence the prognosis in study I (chapter 4) and probably will not influence outcome in study II when the data becomes available (chapter 5). WCC was found not to significantly influence outcome in study I and in study II there were 10 patients with inv(16) and t(16;16), with WCC greater than 30 x 10^9/L, and their survival varied from 10 days to 963 days. However this group did contain all of the early deaths for study II (patients #16, #23 and #26). Only 3 patients in these studies where clinical outcome is known, were over the age of 55 (chapter 4: patient #5 and chapter 5: patients #24 and #26) the last patient at age 65 years had a very poor outcome of 28 days the other two patients survived over 1 year. It is difficult to combine the data for the two groups because the clinical outcomes quoted for the two studies are very different. Therefore only general observations for the comparison of the two groups can realistically be made. However, it would appear that despite a good outcome for the inv(16)/t(16;16) group in general, there must be factors other than the known poor prognostic factors (age, karyotype and WCC) that are influencing survival in this group of leukaemic patients.

The discovery of a deletion associated with the short arm breakpoint in a subgroup of the inv(16) suggested that there would be genes other than *CBFB* and *MYH11* involved. This led to the investigation of the involvement of *MRP* and subsequently *ARA* in the deletion of the short arm. With regard to *MRP*, it was the knowledge that it mapped to 16p13.1 that led to a request to Prof Roger Deeley for an *MRP* probe. The rationale being that if a multidrug resistance gene were deleted in the inv(16) leukaemias, it may help to explain the biology of the disease. The subsequent finding of a correlation between the deletion of *MRP* and the

time to failure of the patients was encouraging. The analysis of the expression of *MRP* in these patients was also encouraging as it revealed significant differences in expression of *MRP* when *MRP* deletion and non-deletion cases were compared. An update of the survival data of the patients in this study is eagerly awaited so that the effects of the expression of *MRP* on overall survival and the duration of first remission/time to failure for these patients can be analysed in detail. The subsequent finding of the deletion of *ARA* in some of the inv(16) deletion cases, in the last month of the thesis work, provided further encouragement that the question of multidrug resistance in the inv(16) leukaemias is an important one to further investigate.

The survival of patients with leukaemia, as has been discussed, is dependent on a number of factors. The relationship of expression of P-glycoprotein and clinical outcome has not been conclusive. However subsequent to the finding of more drug resistant factors expressed in haematopoietic precursors, namely MRP, ARA, Topoisomerases and possibly LRP, it is evident that more comprehensive studies of drug resistance mechanisms and their expression in leukaemias is required. If we are to persist with the current forms of therapy, namely cytotoxic agents, methods of overcoming intrinsic resistance of the leukaemias is urgently needed. Although the results of this form of therapy are encouraging in the paediatric population, the results in the adult population remain generally poor with only 10% of adults (15-60 years) with acute myeloid leukaemia treated with chemotherapy remaining in remission for greater than 5 years. The knowledge of the degree of drug resistance expressed by any one leukaemic population may be able to direct therapy changes for the patient if we are able to demonstrate the relationship between these factors and clinical outcome. These

changes in therapy may incorporate resistance reversing agents to the induction chemotherapy or may influence the decision for the timing of transplantation in a given patient.

The results of the work from this thesis are an important contribution to the knowledge of multidrug resistance and its role in acute leukaemia. However the contribution is but a small drop in the ocean when the amount of information required to directly benefit the patient with leukaemia is considered. This thesis is a personal achievement but it is hoped that the continuance of this work will some day amount to more than this, if in fact there is more than personal achievement that matters at the end of the day.

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APPENDIX

The concept that fetal stem cells may be able to give rise to both haematopoietic and stromal cells is attractive yet the conclusions are controversial. To date there is no conclusive evidence that this occurs. The data presented by Huang and Terstappen in Nature 1992, had to be withdrawn in 1994, as many conclusions had been drawn on morphological grounds rather than immunophenotypic grounds.

Embryonic haematopoietic stem cells are first found in the fetal yolk sac as early as day 8 in the mouse and are then found in the embryo within the fetal liver. The fetal yolk sac gives rise to haematopoietic precursors, endoderm and gonadal cells. The question remains as to whether the bone marrow microenvironment is derived from the yolk sac along with the developing blood cells, with specialised capabilities with respect to blood cell development. The haematopoietic microenvironment provides a source of growth factors for haematopoietic progenitors, some of which are diffusable while others are dependant on contact with the stromal cell layer. Embryonic stem cells cultured as embryoid bodies (EBs) in the absence of exogenous factors can give rise to blood islands and advanced EBs have been identified that give rise to the three germ layers (ectoderm, endoderm and mesoderm) (Chen 1992). There are significant quantitative differences between even yolk sac and embryonal stem cells (Huang 1994), however qualitative differences are more difficult to demonstrate. There are also quantiative differences between human umbilical cord blood and adult bone marrow, cord blood being a rich source of haematopoietic precursors, CD34+ cells (Lansdorp 1994).

Singer 1984 suggested a common stem cell on evidence from patients with clonal haematopoiesis: CML and AML. An early paper by Islam 1988, suggested direct evidence for a stem cell common to both haematopoiesis and stromal development, however its data was flawed in that although only adherent cells were transfused into irradiated rats, this layer

may well have harboured adherent haematopoietic precursors capable of reconstituting the bone marrow of the irradiated rats. More sophisticated means of identifying haematopoietc and stromal precursors are now available. Waller (1995b), examined flow sorted $CD34^{+}$ $CD38^{-}$ $HLA\text{-}DR^{-}$ cells and demonstrated that cells from this group could develop along haematopoietic and stromal lines and that the numbers of stromal progenitors present in this population of cells diminished with fetal maturation (Waller 1995a), however when sorted into single cell chambers, dual lineage differentiation could not be demonstrated.

It is well documented (Simmons 1991b) that stromal precursors express CD34 antigen, a 115kDa glycoprotein that is well established as a differentiation antigen expressed on primitive myeloid and lymphoid progenitors (Katz 1985, Andrews 1986). Simmons demonstrated the expression of CD34 and STRO-1 (MoAb recognizing a stromal cell antigen) on CFU-F (fibroblast). He also demonstrated that LTBMC stromal cells can be generated from CD34+ selected marrow cells confirming that both the haematopoietic microenvironment and the haematopoietic precursors themselves can be generated from this population of cells. However, the phenotype of cells which result in CFU-F differs from that which result in haematopoietic colonies: in the expression of STRO-1 and the presence of binding sites for the lectin SBA. In another study (1991a) Simmons demonstrated that 95% of the STRO-1 positive cells in the bone marrow were in fact nucleated erythroid precursors. However, it was demonstrated that STRO-1 failed to bind to T cells, B cells, myeloid cells, macrophages or megakaryocytes. These studies demonstrate some overlap in expression of antigens by both haematopoietic and stromal cells however the significance of the expression of CD34 on stromal cells remains to be determined. Whether it represents some common ancestry remains to be ellucidated . To date there is no conclusive evidence that supports the hypothesis of a common ancestry for haematopoietic cells and stromal cells.

MINOR AMENDMENTS

p2 The sentence at the end of the paragraph should be altered: For example, the immunophenotype $CD34^{+}CD38^{-}$ lin^{-} DR^{-} present in human fetal marrow can give rise to lymphoid and haematopoietic cells and may give rise to a LTC-IC type (Verfaillie 1990).

p12 L12 localise not localised

p14 L10 delete required

p17 L3 The *MDR1* gene is the human type I P-glycoprotien gene, the mouse has two such isoforms not one.

p17p2 L1 ...is a transmembrane region

p19 L16 versus

p22 p2 Delete "*in situ* hybridisation studies and"

p23 HL60 should be referred to as a myeloid leukaemia cell line capable of differentiation following exposure to retinoic acid and other agents.

p27L15/16 The proteins separately referred to as LRP and the 110kDa protein are one and the same, now more frequently known as LRP. The more appropriate reference is Scheper 1993.

p28 L6 This correlates to replace Therefore correlating, at the beginning of the sentence.

p52-2.3.3.2 Polyacrylamide

p52 L6 gel trays to replace casts

p56 L1 Oligolabelled probe refers to a probe labelled using random oligonucleotide primers.

p119 L11 add "...where the alpha subunit of the CBF was critical for the T-cell specificity"

p128 (gr). Patient#9 who was in relapse

p186 The highest ratios were seen in patients #23 and #27 not #22 and #26 as stated in the text.

p196 The last few lines state that a silencer element is present in the *MRP* promoter region. This was subsequently reported to be a cloning artifact.

p220 Refers to "the student" which is me (Bryone J Kuss).

legend to Figure 1.1 line 6 give rise to immediate and short-lived repopulation

legend to Figure 3.9 line 5 drigest should be replace with DRIgest TMIII, Pharmacia

legend to figure 4.7 D16S49 is represented by panel A and D16S405 is represented by panel B. Patient 9 is figured in lanes 8 and 19.

Table 5.5 The fusion transcripts known and reported by Dr Willman's group are: Pt.#1 - transcript A; Pt.#2 - transcript A; Pt.#3 - transcript A; Pt.#4 - transcript A; Pt.#5 - transcript F; Pt.#6 - transcript A; Pt.#7 - transcript E; Pt.#16 - transcript A; Pt.#19 - transcript A; Pt.#22 - transcript A; Pt.#23 - transcript A; Pt.#25 - transcript A. The transcripts are as listed in table 3.14.